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*The William Townsend Porter
Memorial Volume*

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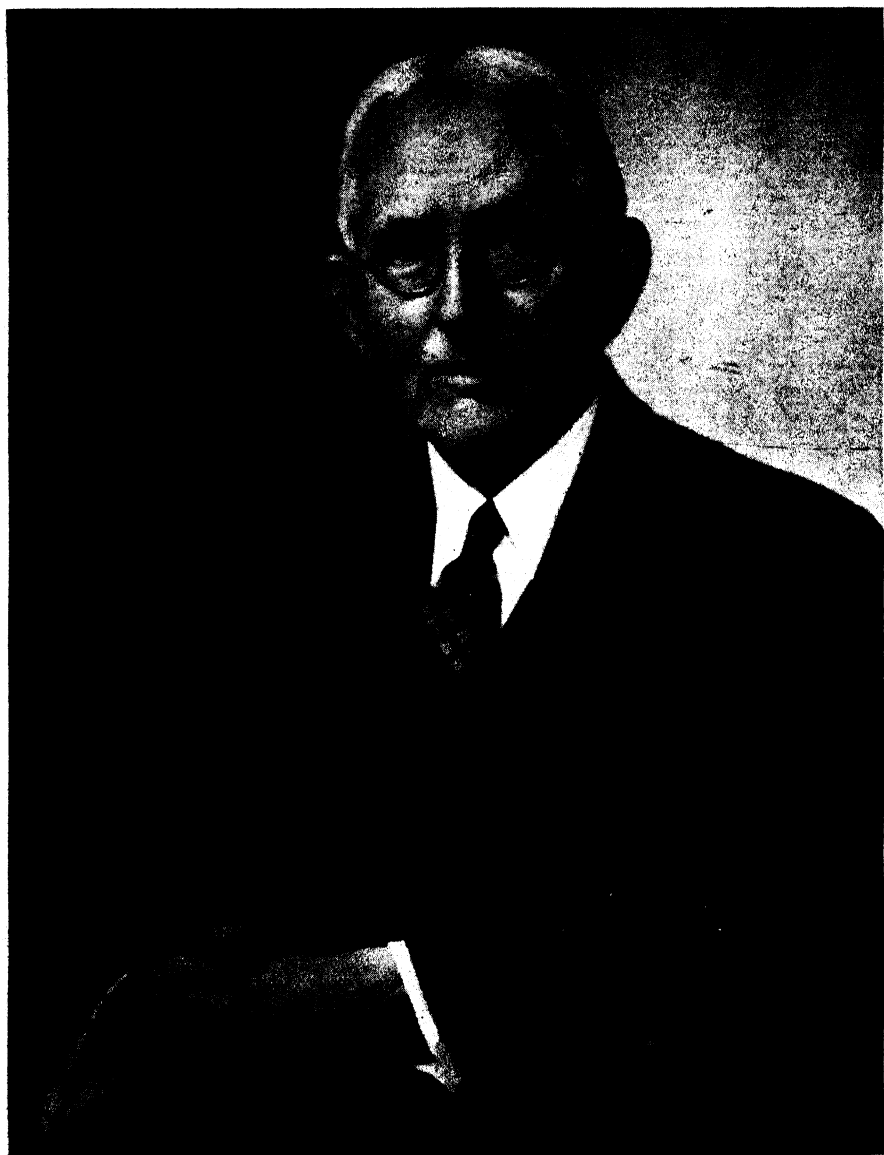
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He believed that a meritorious discovery may fail of appreciation because of the faulty manner in which it is announced to the world, and that an editor may be of service to an investigator. He believed that a scientific journal, the organ of a national science, should be characterized by scientific merit, rhetorical excellence, and prompt publication of its contributions, together with typography and illustrations that are pleasing to the eye. These ideals he has maintained.

AMERICAN JOURNAL OF PHYSIOLOGY
Volume 37, 1915—Dedication
to William Townsend Porter





WILLIAM TOWNSEND PORTER

1862-1949

Editor of THE AMERICAN JOURNAL OF PHYSIOLOGY, 1898-1914

THE RAPID DEVELOPMENT of physiology in the United States could be described very adequately by the lives of a small company of leaders each of whom contributed, during the period of 1890 onwards, his own complement of enthusiasm and particular talent. In this group Professor William Townsend Porter will always hold a major position. It was his keen foresight and self-sacrificing zeal that identified and provided several of the elements so essential for a growing science.

William Townsend Porter was born on September 24, 1862, in Plymouth, Ohio, the son of Dr. Frank Gibson Porter and Martha Townsend Porter. After receiving his doctorate in Medicine in 1885 from the St. Louis Medical College (later incorporated into Washington University), he spent a year in post-graduate study abroad under the tutelage of Flemming, Heidenhain, and Hürthle. In 1888, at the age of 26, he was appointed Professor of Physiology in the St. Louis Medical College and established at once what he called, with justifiable pride, the first physiological laboratory west of the Atlantic seaboard. Publications from this laboratory, appearing in British, German and American journals, promptly drew attention to the youthful William Porter. Called to Harvard Medical School under Professor Bowditch he was an Assistant Professor of Physiology from 1893 to 1898, Associate Professor from 1898 to 1906, and then Professor of Comparative Physiology until retirement in 1928, when he was made Professor Emeritus.

During his first few years at Harvard, while sending to journals of physiology abroad the results of his numerous studies on respiration and circulation, he repeatedly stressed, verbally and in writing, the urgent need for developing in this country a suitable medium for publishing the results of physiological research. When the young American Physiological Society debated from 1894 to 1898 the feasibility of establishing its own journal, it was W. T. Porter who resolved the problem by volunteering to undertake not only the managing editorship, but also full financial responsibility. To his activities as an investigator and teacher he added those of a zealous editor and astute business manager. The need for the journal can best be estimated by examining the contents of the first volume, which was published in 1898 and included papers by Porter, Howell, Lusk, Mendel, Cushney, Chittenden and A. N. Richards, together with Walter B. Cannon's paper on the use of x-rays to study gastric motility.

Having edited thirty-three volumes during the next sixteen years, W. T. Porter in 1914 turned over to the American Physiological Society an established journal of high standards and proved scientific value. In 1915 volume 37 of the *AMERICAN JOURNAL OF PHYSIOLOGY* was dedicated to him with these concluding words of tribute: "For his unselfish labors Professor Porter deserves the thanks of American physiologists and as an expression of this gratitude, they gladly dedicate to him this volume."

During this same period physiological research and teaching were hampered by the necessity of importing physiological instruments from Europe at costs which were prohibitive for many of the younger and newly established American laboratories. Professor Porter's devotion to physiology, together with his love of perfection in experimental technique and reasoning, led him again to use his own time, ingenuity and resources to the benefit of physiology and physiologists in general. In 1901 he established the Harvard Apparatus Company, an independent, non-profit organization dedicated to the "advancement of laboratory teaching in physiology and allied sciences." Instruments made by this Company's skilled mechanics can be found now in research and teaching laboratories the world over. In 1929, the Council of the American Physiological Society again expressed their debt to Professor Porter by recording that "there is no one agency, during recent years, which has contributed more to the development of sound teaching in experimental physiology in this country than the Harvard Apparatus Company."

Professor Porter realized that however good laboratories and journals may be, research depends finally and essentially upon trained investigators. To this end he made still another outstanding contribution. Because he would not accept any salary from the Harvard Apparatus Company, this avocation of his produced by 1920 an annual surplus which he devoted to the establishment of a Research Fellowship in Physiology. He entrusted the choice of Fellows and administration of the Fellowship to the American Physiological Society which named it, in his honor, the Porter Research Fellowship in Physiology. His philanthropy, using that term in its most literal sense, has made it possible, since 1921, for over a score of outstanding young students to devote a year or more to research in laboratories of physiology under experienced preceptors. This group already includes several professors and winners of distinguished prizes for outstanding research. Up to the last moment before his final illness and death on February 16, 1949, Professor Porter was trying to assure, through this Fellowship, continued funds for training young investigators. By the death of Professor William Townsend Porter, Physiology has lost not only an investigator and teacher whose researches advanced knowledge but also a benefactor whose influence will long be felt through the Research Fellows whose training in research he has made possible.

Among the honors which came to him, the one he appreciated most must have been his election as Honorary President of the American Physiological Society in 1937 on the occasion of its fiftieth annual meeting. The words by which he was introduced at the Semicentennial Dinner describe Professor William Townsend Porter's ruling passion and devotion. "Few indeed are the men who have had American Physiology so near the heart." The reality of this devotion he expressed in his own words in a recent letter: "To me Physiology is a religion. The AMERICAN JOURNAL OF PHYSIOLOGY, the Fellowship of the American Physiological Society, and the Harvard Apparatus Company are votive candles, burning with a steady flame on the high altar of a faith."

The American Physiological Society expresses once more its gratitude to a benefactor, friend and distinguished Honorary member by dedicating this volume to William Townsend Porter, Professor of Comparative Physiology, Emeritus, Harvard Medical School, and by listing the names of those who have held the Porter Research Fellowship from 1921 to the present.

—EUGENE M. LANDIS, 1949

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1922-23	Florence B. Seibert	A.B., Goucher	Yale	Lafayette B. Mendel	Physiological actions of proteins
1923-24	Florence B. Seibert	Ph.D., Yale	Chicago	H. Gideon Wells	" "
1924-25	Howard H. Beard	A.M., Columbia; Ph.D., Yale	Yale	Lafayette B. Mendel	Metabolism
1925-26	W. H. Finney		McGill	John Tait	Nitrogen metabolism, hibernation
1926-27	Charles Stuky	A.B., Ph.D., Yale	Yale	Lafayette B. Mendel	Metabolism and vitamin B
1927-28	Dea B. Calvin	B.S., Rice Institute; Ph.D., Yale	Yale	Lafayette B. Mendel	Blood sugar
1928-29	Donald E. Gregg	B.S., Colgate	Rochester	John Murlin	Fat and sugar metabolism
1929-30	Donald E. Gregg	Ph.D., Rochester	Rochester	John Murlin	" "
1930-31	Herbert Silvette	B.S., Virginia	Virginia	Sidney W. Britton	Endocrines
1931-32	Herbert Silvette	Ph.D., Virginia	Virginia	Sidney W. Britton	" "
1932-33	Abraham White	B.A., Denver; M.A., Ph.D., Michigan	Yale	Arthur H. Smith	Acid-base equilibrium
1933-34	Nathan Rakieten	B.S., Wesleyan; Ph.D., Yale	Yale	John Fulton	Central nervous system
1934-35	Edward H. Kemp	B.A., Wake Forest	Clark	Walter S. Hunter	Auditory reactions
1935-36	Ellen Robinson	B.S., Radcliffe	Harvard	Alfred C. Redfield	Auditory action potentials
1936-37	H. C. Wiggers	A.B., Wesleyan; Ph.D., Western Reserve	Harvard	Hallowell Davis	Auditory action potentials
1937-38	Jane A. Russell	A.B., Ph.D., California	California	Hervert M. Evans	Pituitary and sugar metabolism
1938-39	Earl R. Loew	B.S., Michigan State; M.S., Wayne	Northwestern	A. C. Ivy	Duodenal hormone and sugar metabolism
1939-40	N. S. R. Maluf	B.S., American (Cairo); Ph.D., Cornell	Washington	H. L. White	Renal physiology
1940-41	Gordon K. Moe	B.S., M.S., Ph.D., Minnesota	Western Reserve	C. J. Wiggers	Heart and circulation
1940-41*	J. H. Wills	B.S., Va. Polytech. Inst.; M.S., Medical Coll. of Va.; Ph.D., Rochester	Harvard	W. B. Cannon	Gastro-enterology
1941-42	Milton J. Schiffrin	A.B., M.S., Rochester; Ph.D., McGill	Northwestern	A. C. Ivy	Ulceration of gastro-intestinal tract
1942-45 Porter Fellowship temporarily suspended					
1946-47	E. L. Chambers	B.A., Princeton; M.D., New York	California	S. C. Brooks	Permeability to radioactive ions
1947-48	Arthur F. Battista	M.D., McGill; M.S., Western Ontario	Harvard	Alexander Forbes	Neurophysiology
1948-49	Raymond F. Kline	B.S., M.S., Virginia	Maryland	W. R. Amberson	Anoxia
1949-50	Philip W. Hall, III	B.S., Bethany	Western Reserve	C. J. Wiggers	Renal vascular physiology

* Supplementary Fellowship

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HYDROLYSIS OF CHOLINE ESTERS IN THE PRESENCE OF ADRENALIN

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THE inhibition of cholinesterase activity by adrenalin was reported recently (1). The esterases used in these studies represented what were termed 'specific' and 'non-specific' cholinesterases. They were obtained, respectively, from the caudate nucleus and the parotid gland of the hog. The substrate used was acetylcholine.

It was considered desirable to study further the inhibitory effect of adrenalin using additional substrates and cholinesterase of other sources. This paper reports upon the findings of these studies.

METHODS

The Warburg manometric technic was employed, following the procedure outlined in the previous publication (1). Two of the enzymes consisted of preparations of cholinesterase obtained from the caudate nucleus and the parotid gland of the hog. A third esterase, one also obtained from the parotid gland, differed from the one described above in that it was brought to dryness over calcium chloride rather than lyophilized in the final stage of preparation. All of these preparations were prepared in part similar to the technic of Mendel and Mundell (2) for the purification of a pseudo cholinesterase from dog pancreas.

In addition to these, the fresh frozen caudate nucleus of the dog was used for a further comparison of enzyme activity. The latter tissue was homogenized and diluted with Krebs-Henseleit (3) buffer solution. The activity of varying but known quantities of this tissue, approximating 5 mg. of wet weight, was followed in individual Warburg flasks. The tissue hydrolyzed acetylcholine

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and metacholine (acetyl-beta-methylcholine) readily. It also hydrolyzed benzoylcholine to a minor extent but was considered similar to the more purified hog caudate nucleus as representative of the 'specific' type of cholinesterase. The parotid preparations were of the 'non-specific' type. All of the enzyme preparations were dissolved or suspended in Krebs-Henseleit solution.

The substrates, consisting of acetylcholine, methacholine and benzoylcholine, were made up in buffer solutions. They were added in 0.5 ml. amounts (to make a final 0.003 molar concentration) to a side arm of their respective double sidearm flasks. Adrenalin hydrochloride, prepared from its base, was added in 0.5 ml. amounts to the other side arm.

Three concentrations of adrenalin were studied: 0.0015, 0.003 and 0.006 moles/liter. Immediately after placing the adrenalin in the side arms of the Warburg flasks, the flasks and the manometers were gassed with a mixture of 5 per cent CO_2 -95% N_2 to prevent its oxidation. After equilibration at 37.5°C. the contents of the side arms were tipped into the reaction chamber. The addition of adrenalin preceded that of the substrates. Controls and blanks, including those for non-enzymatic hydrolysis, were run simultaneously.

Gas evolution, resulting from the liberation of acetic acid and its action on bicarbonate, was measured in the conventional manner. All experiments were run in duplicate. The results were expressed in microliters of CO_2 released per mg. of dry weight of tissue in 60 minutes of time.

RESULTS

The results of these studies are presented in table 1. As was reported previously, the ability of cholinesterase to hydrolyze acetylcholine is inhibited by the presence of adrenalin. This is true regardless of the source or type of esterase. With increasing concentrations of adrenalin, the activity is increasingly depressed. Enzyme from the caudate nucleus of the hog and dog, acting upon methacholine, is inhibited in a similar manner. Inhibition also occurs when the parotid gland preparations act upon benzoylcholine in the presence of adrenalin.

The percentage inhibition of the 4 cholinesterase preparations is presented in table 2. The values are based upon the averages of the duplicate determinations of table 1. The inhibition of cholinesterase by adrenalin is greater when acting on methacholine than when it is acting on acetylcholine. This is demonstrable with both the lyophilized preparation of the hog's caudate nucleus and the homogenized preparation of the dog's caudate nucleus. Conversely, the inhibition of cholinesterase by adrenalin is less when the esterase is acting on benzoylcholine than when it is acting on acetylcholine. This is evident with both the lyophilized and the calcium chloride-dried preparations of the parotid gland. These relationships exist at all concentrations of the inhibitor.

This finding is more striking when it is appreciated that the degree of inhibition is not related to the rates at which the cholinesterases hydrolyze a given choline ester. A comparison of the lyophilized preparation of the hog's caudate nucleus (which represents a slow rate of catalysis) and the homogenized preparation of the dog's caudate nucleus (which represents a fast rate) bears this out. The degree of inhibition is similar whether normal catalysis is slow or fast.

The activity of two different types of esterases, the homogenized 'specific' esterase of the dog's caudate nucleus and the lyophilized 'non-specific' esterase of the hog's parotid gland, fortuitously, hydrolyzed acetylcholine at the same rate. It is, therefore, possible to compare directly the rates at which the one enzyme metabolizes methacholine and the other metabolizes benzoylcholine. It may be seen from table 1 that their activities in this respect are equivalent. In plotting the average percentage

TABLE 1. ACTIVITY OF VARIOUS CHOLINESTERASE PREPARATIONS IN THE PRESENCE OF ADRENALIN¹

PREPARATION	SUBST. ²	CONTROL	CONCENTRATION OF ADRENALIN		
			0.0015M	0.003M	0.006M
Caudate nucleus (Lyophil.; hog)	Ach	39.4	33.5	30.4	24.5
		36.5	33.3	28.4	23.4
	Mch	5.2	3.9	2.5	1.9
		5.0	3.1	1.9	0.7
Caudate nucleus (Homog.; dog)	Ach	206.5	200.9	145.7	126.3
		198.8	156.4	136.4	126.2
	Mch	60.4	46.9	40.8	32.5
		60.2	44.4	40.6	30.7
Parotid gland (Lyophil.; hog)	Ach	205.4	172.9	147.7	109.7
		201.4	171.7	147.0	106.0
	Bch	61.0	61.8	53.5	47.8
		60.8	58.7	52.6	45.8
Parotid gland (dried; hog)	Ach	217.6	194.3	172.7	140.1
		214.8	190.7	169.8	127.6
	Bch	74.5	65.1	64.4	58.1
		71.6	67.0	63.3	56.2

¹ Activity is expressed in microliters of CO₂ released in 60 min. of time per mg. dry wt. of preparation.

² Ach, Mch and Bch denote the substrates acetylcholine, methacholine and benzoylcholine in 0.003M concentration.

TABLE 2. PERCENTAGE INHIBITION OF CHOLINESTERASE BY ADRENALIN¹

PREPARATION	CONCENTRATION OF ADRENALIN					
	0.0015M		0.003M		0.006M	
	<i>Ach</i>	<i>Mch</i>	<i>Ach</i>	<i>Mch</i>	<i>Ach</i>	<i>Mch</i>
Caudate nucleus Lyophil.; hog	12.1	31.0	22.7	56.5	36.9	74.5
Homog.; dog	11.8	24.3	30.4	32.5	37.7	47.6
<i>Average</i>	12.0	27.7	26.6	44.5	37.3	61.1
Parotid gland Lyophil.; hog	15.3	1.1	27.6	12.9	47.0	24.1
Dried; hog	10.9	9.6	20.8	12.6	38.1	21.8
<i>Average</i>	13.1	5.4	24.2	12.8	42.6	23.0

¹ Percentage inhibition is based upon averages of activities expressed in table 1.

of cholinesterase inhibition against the concentrations of inhibitor agent, as was done in figure 1, it is obvious that a marked difference in the two enzyme systems exists.

With methacholine as the substrate for the 'specific' type of esterase, the degree of adrenalin inhibition is high. With benzoylcholine as the substrate for the 'non-specific' esterase, the degree of inhibition is low.

DISCUSSION

The cholinesterases, in general, have been considered as a group of related and yet individual enzymes (4-6). They have been classified on the basis of the relation of their activity to the substrate concentration (5-8). The one type is characterized by maximum hydrolysis of acetylcholine at about 3×10^{-3} molar concentration. Inhibition occurs at concentrations above this. The other type is characterized by maximum hydrolysis at infinite substrate concentration. Unfortunately, the group numbers used by Augustinsson (6) are just the reverse of those used by Bodansky

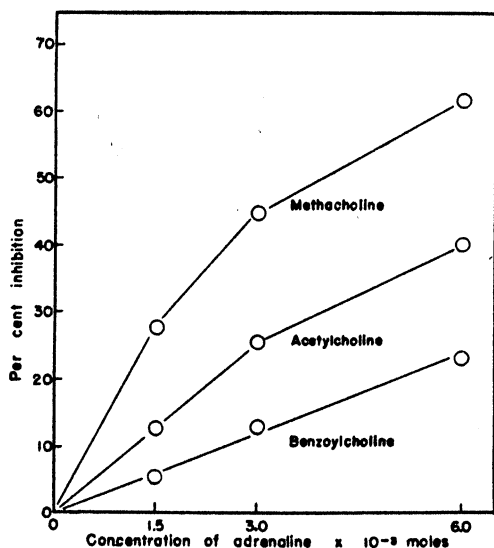


Fig. 1. INHIBITION OF CHOLINESTERASE by varying concentrations of adrenalin

(5). The enzymes of the caudate nucleus and the parotid gland used in this report correspond respectively to the above two groups. To avoid further confusion by numbering them, and yet to indicate the type of esterase to which they have been considered to belong in the past, the terminology of 'specific' and 'non-specific' cholinesterases has been retained in this paper. In view of the fact that both types of esterases are able to hydrolyze non-choline esters (5, 9) it may be expedient in the future to refer to these as *Groups A* and *B*, respectively.

The low order of activity of the lyophilized preparation of the caudate nucleus of the hog is of interest. This material lost over 50 per cent of its original activity when stored at 4°C . for a period of 2 months. It was also readily inactivated by heat. This is in marked contrast to the parotid preparations, which were only partially inactivated when heated at 110°C . for 24 hours. The 'specific' type of esterase is thus more readily inactivated than the 'non-specific' type. Studies on the thermolability and relative thermostability of the two esterases will be reported elsewhere.

It is of considerable interest that the two different esterases are inhibited by adrenalin to a similar degree when acting upon acetylcholine but to markedly different degrees when acting upon their respective substrates, methacholine and benzoylcholine. Since Augustinsson (6) has shown with dog's brain that the substrate concentration for optimum hydrolysis of acetylcholine and methacholine are very nearly the same, the difference in the degree of inhibition with different substrates suggests that the process involves factors other than competition for reactive groups. Preliminary studies indicate that the inhibition is non-competitive in nature.

SUMMARY

Adrenalin in concentrations varying from 0.0015 to 0.006 moles/liter inhibits the ability of cholinesterase of various sources to hydrolyze choline esters in 0.003 molar concentrations. With acetylcholine as the substrate, the degree of inhibition of 'specific' and 'non-specific' cholinesterases is of the same order of magnitude. With methacholine as the substrate for the 'specific' esterase, the degree of inhibition is comparatively high. With benzoylcholine as the substrate for the 'non-specific' esterase, the degree of inhibition is relatively low.

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INACTIVATION OR REMOVAL OF INSULIN BY THE LIVER¹

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IT WAS pointed out by Collens and Murlin (1) and Neuwirth, Co Tui and Wallace (2) that if one judged the effect of insulin by means of change in the blood sugar, then the intraportal injection of the hormone was less effective, unit for unit, than the injection into a peripheral systemic vein. These same authors, as well as Burger and Kramer (3), also found that a temporary initial rise in blood sugar is more frequently observed after intraportal insulin injections (single or repeated), than after injections via a systemic vein.

The realization that samples of insulin contain variable amounts of a hyperglycemic, glycogenolytic factor (H.F.) (4) might lead to the assumption that the intraportal injection of insulin was only apparently less effective, the expected hypoglycemia being masked by a simultaneous glycogenolytic action of the H.F. On the other hand, the intraportal route may be less effective because insulin itself may be partially inactivated or otherwise changed by its passage through the liver (5-7).

We tested these two possibilities by comparing the effectiveness of equal amounts of insulin injected continuously via the femoral vein and the splenic vein in normal and depancreatized animals. The participation of the hyperglycemic factor was tested by using two brands of insulin—one containing, and the other free of H.F.

METHODS

All experiments were done on dogs under pentobarbital anesthesia. Depancreatized animals were used after a 72-hour period free of food and insulin. Comparisons between intrasplenic and intrafemoral injections were made on the same animal at 3-day intervals. The sequence of injection routes was varied and had no apparent effect on the results.

Preliminary experiments were done with the dose of insulin varying from 1/5 to 1/50 U/kg/hr., given as a constant injection in 52 cc. of saline per hour for 2 hours. The experiments reported were done with 0.1 U/kg/hr. since this dose gave definite drops in blood sugar via both injection routes. Arterial blood samples were analyzed in duplicate for glucose by the Somogyi modification (8, 9) of the Shaffer-Hartmann Technique.

RESULTS

Our preliminary experiments revealed that there was a gradation of effect of the injected insulin. The minimum effective dose was found to lie between 1/50 and 1/25 U/kg/hr.

Figure 1 presents the results in normal dogs injected with 0.1 U/kg/hr. of Lilly insulin, known to contain H.F. (4). It can be seen that there is a small but definite

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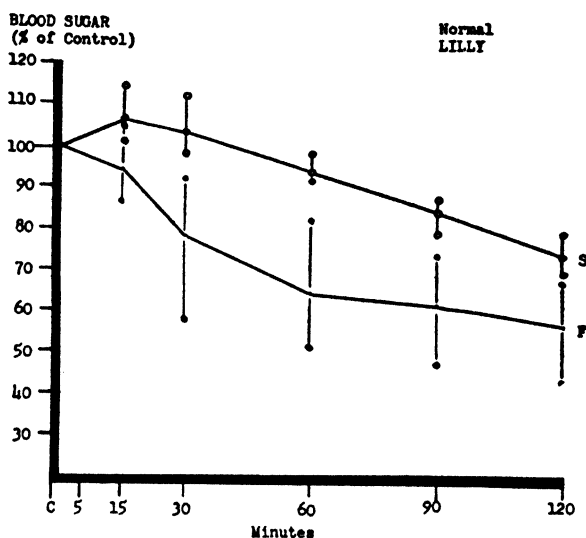
² The department is supported in part by the Michael Reese Research Foundation.

initial rise during the first 15 minutes of the splenic infusion. The rate of fall between 15 and 60 minutes was greater when the femoral route was used. The net result is that the femoral route gives a significantly greater blood sugar depressing effect than does the splenic route. Despite absolute variations, this was the case in each animal used.

Essentially the same results were obtained when these experiments were done on 2 totally depancreatized dogs (fig. 2). The fasting blood sugar levels of these animals varied from 272 to 519 mg.%, but in order to compare the results with the normal group, the curves are plotted as percentage change from the pre-injection value.

Figure 3 presents similar data on 2 normal dogs given the same amount of insulin (Novo) not containing any appreciable amount of H.F. It can be seen that the blood sugar depressing effect of this insulin is significantly greater than that of the previous

Fig. 1. EFFECT OF 0.1 U/KG/HR. of Lilly insulin in normal dogs. *Upper curve:* splenic vein injection (3 exper.). *Lower curve:* femoral vein injection (7 exper.). Range of values shown for each curve.



brand, by both routes of administration. However, even in the absence of demonstrable H.F., the difference in the effectiveness of the femoral *vs.* the splenic routes is preserved.

Table 1 expresses the insulin effect in terms of the area of the curve. The ratio of effect of the splenic route to the femoral route is seen to be 1:3.85, 1:1.82 and 1:1.83 for the three sets of experiments.

DISCUSSION

It is apparent from the data presented that the lessened effectiveness of a given dose of insulin when injected intraportally as compared to an intrafemoral injection cannot be completely ascribed to the masking action of a glycogenolytic effect of an admixed H.F. Comparison of figures 1 and 3 shows that there is a greater fall in each curve when Novo insulin is used. The data in table 1 show that the femoral route is 3.85 times as effective for Lilly insulin in contrast to 1.83 times for Novo. This

may be explained by the presence of the H.F. which causes the liver glycogen to fall and the blood sugar to rise, thus resulting in a small drop in the blood sugar when insulin is given to normal animals via the splenic vein.

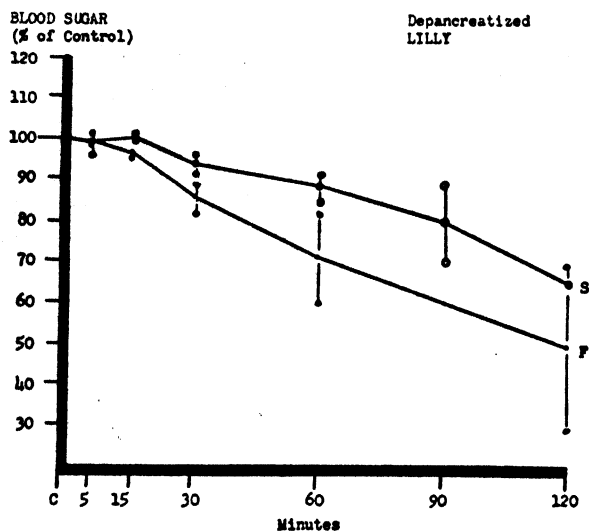


Fig. 2. EFFECT OF 0.1 U/KG/HR. of Lilly insulin in 2 totally depancreatized dogs. Upper curve: splenic vein injection. Lower curve: femoral vein injection.

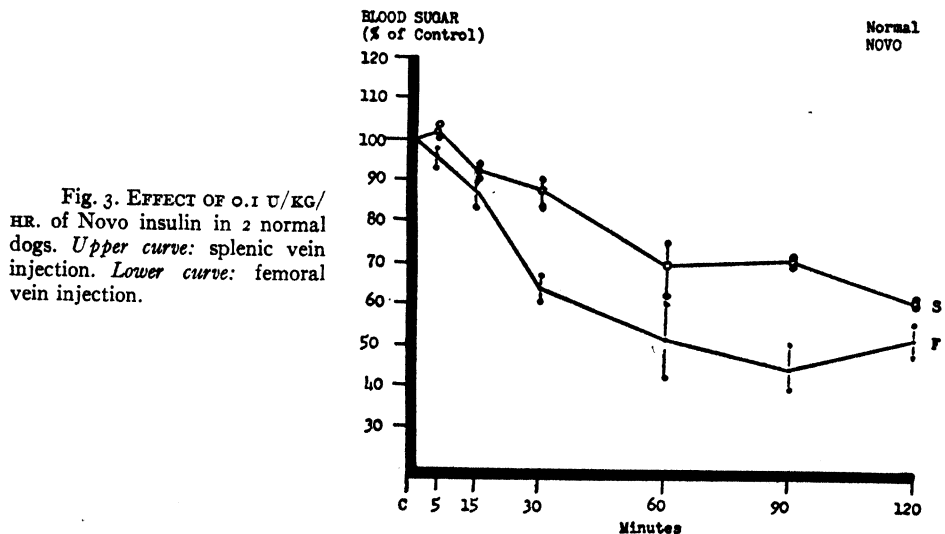


Fig. 3. EFFECT OF 0.1 U/KG/HR. of Novo insulin in 2 normal dogs. Upper curve: splenic vein injection. Lower curve: femoral vein injection.

The liver apparently either retains or inactivates a portion of the insulin which comes to it through the portal vein. This is consistent with the findings of Broh-Kahn and Mirsky of the presence of an enzymatic system ('insulinase') in the liver capable of destroying the insulin molecule (5-7). They reported that liver contains the greatest 'insulinase' activity; kidney and muscle less. The enzyme system was found in the liver of the rat, rabbit, steer, chicken and man.

Insulinase will explain the differences observed in splenic *vs.* femoral injection. Despite the effect of the H.F. the inactivation or removal of insulin by the liver is seen even when Novo insulin was used. The area difference between splenic and femoral (table 1) is 22.3 and 18.8 for Lilly and Novo insulin, respectively. The difference for the depancreatized animals is 12.1.

Broh-Kahn and Mirsky (7) showed that fasting causes a reduction in insulinase activity of the liver. This explains the results obtained in our depancreatized dogs which were without food for 72 hours. Deprivation of food for this period of time is sufficient to cause a decreased liver insulinase activity so that less insulin would be inactivated. This is seen in the 7.8 and 14.7 units of area for the normal and depancreatized splenic injections, respectively. These two groups did not fall as low as the Novo group (22.6) because of the presence of the H.F. The decreased insulinase activity is also demonstrated in the small (12.1) difference in area between splenic and femoral injections in the depancreatized group.

Our screening experiments showed that $1/25$ to $1/50$ U/kg/hr. had very little effect in lowering the blood sugar. This compares favorably with Slater *et al.* (10)

TABLE 1

CONDITION OF ANIMALS	TYPE OF INSULIN	AREA ¹		DIFFERENCE	RATIO OF EFFECT Splenic:Femoral
		Splenic	Femoral		
Normal	Lilly	7.8	30.1	22.3	1:3.85
Depancreatized	Lilly	14.7	26.8	12.1	1:1.82
Normal	Novo	22.6	41.4	18.8	1:1.83

¹ By 'area' is meant the area enclosed by the blood sugar curve and the horizontal line passing through 100 in figs. 1, 2, and 3, expressed in arbitrary units.

who found that a single intravenous dose of $1/40$ U/kg. in normal dogs caused either no change or only a slight temporary drop in blood sugar.

It may be that the dog liver insulinase is capable of inactivating about $1/25$ to $1/50$ U/kg. body wt/hr. of insulin.

SUMMARY AND CONCLUSIONS

Under normal circumstances insulin, after leaving the pancreas, reaches the liver before it goes into the general circulation. Our evidence shows that the liver removes or inactivates insulin as it passes through the liver. This is probably via the 'insulinase' system described by Mirsky and Broh-Kahn. Liver inactivation of insulin is demonstrated in normal and depancreatized animals. This action is found despite the presence of the hyperglycemic factor in certain insulin preparations. It is conjectured that the liver of the dog can inactivate about $1/25$ to $1/50$ U/kg. body wt/hr. of insulin.

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SEASONAL CHANGES IN THE THYROID GLAND AND EFFECTS OF THYROIDECTOMY IN THE MALLARD, IN RELATION TO MOULT

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A NUMBER of observers have shown that the administration of thyroid or thyrotropic preparations can bring on a moult outside the normal moulting season in domestic fowl (1-3). Others have shown that thyroidectomy can postpone the moult in chicks (4). Thyroidectomy has also been shown to postpone the moult or to abolish it altogether in a number of other species of birds (5, 6).

These findings have naturally led to the supposition that in birds generally increased thyroid activity is responsible for the shedding of the worn plumage and its replacement by new feathers, which constitute the moult. However, it does not appear that this supposition has yet been proved conclusively in the case of any one species. The findings in anserine birds (swans, ducks and geese) in particular are not in agreement with the view that thyroid activity is responsible for the moult in this group. The observers who have administered thyroid preparations to ducks agree that a moult is not precipitated in these birds by doses which produce this effect in fowls (7-9). Further, Chu (10), referring to unpublished work and therefore without giving further details, reports that adult mallards after complete thyroidectomy still moulted into the next plumage. On the other hand, Voitkewitsch (6) found that thyroidectomy in young ducklings prevented the moult from down into the first true plumage if the operation was performed well before the expected onset of the moult. It should also be pointed out that there is fairly convincing evidence that the onset of the moult in the mallard is controlled by the gonads, since a number of observers have found an indefinite postponement of the moult after castration (see review in 10); Chu was able to bring on a moult by administration of pituitary gonadotrophins.

The work here reported is an attempt to clarify the rôle of the thyroid in the moulting process of the mallard. The mallard has two annual moults. The first takes place in June to July in males; in this the entire plumage is renewed in the change from the mating- or winter- to the eclipse-plumage of summer. In females this moult is delayed to July to August (until the young are partly fledged). A second less extensive moult occurs in the fall; in males August to November, in females October to March. This moult affects the body plumage and only a few of the larger feathers and may be spread over a long period. Moulting periods as given apply to English mallards (16) such as were used for this work. A study of seasonal changes in the thyroid in relation to these plumage changes seemed of particular interest, since on the supposition of a thyroid control of the moult a period of increased thyroid activity would be expected to precede the two moults. In view of the thyroidectomy experiments of Chu and Voitkewitsch, it appeared desirable to investigate the effect of this operation on the moult in somewhat older birds than those used by the second observer.

TABLE I. CONDITION OF THYROID GLAND IN RELATION TO PLUMAGE

DATE	THYROID WT. MG. ¹	THYROID STATE	PLUMAGE TYPE
<i>A. Males</i>			
1/11	110	Intermed.	Mating
1/15	87	Active	Mating
2/15	128	Storage	Mating
3/15 ²	115	Storage	Mating
4/11	45	Active	Mating
5/15	187	Active	Mating
5/24	135	Active	Mating
6/7	114	Storage	Early moult
6/19	75	Storage	Moult
6/21	170	Storage	Moult
7/8	90		Moult
7/16	135	Active	Moult
7/19	150	Storage	Moult + eclipse
7/31	81	Storage	Eclipse
8/2	220	Storage	Eclipse
8/10	100	Storage	Eclipse + 2nd moult
8/13	107	Intermed.	Eclipse + 2nd moult
9/17 ³	89	Active	Nearly full mating
10/15	135	Active	Mating
10/23	95	Active	Mating
11/15	108	Active	Mating
11/20	70	Storage	Mating
11/20	135	Active	Mating
11/23	135	Intermed.	Mating
11/28	80	Active	Mating
11/29	70	Active	Mating
12/11	110	Intermed.	Mating
12/12	88	Active	Mating
<i>B. Females</i>			
1/18	59	Intermed.	Mating + nest down growing
2/19	140	Intermed.	Mating + nest down present
2/22	110	Active	Mating + nest down growing
3/22	113	Storage	Mating
4/24	107	Storage	Mating
5/19	160		Mating
5/24	122		Mating
5/25	126	Intermed.	Mating
6/7	133	Active	Mating
6/21	40	Active	Mating
6/26	72	Active	Mating
7/8	120	Storage	Buds of Eclipse
7/19	107	Storage	Buds of Eclipse
8/2	155	Intermed.	Eclipse
8/10	90	Intermed.	Eclipse
10/30	100	Active	Mating
11/15	—	Active	Mating
12/18	95	Active	Mating

¹ Both lobes. ² This bird had a dermatitis with loss of feathers on the neck. ³ This bird showed signs of a chronic parercatitis.

SEASONAL CHANGES IN THE THYROID GLAND

Material and Methods. Twenty-eight adult male and 16 adult female mallards (*Anas platyrhynchos* L.) were shot at regular intervals in one of the London Royal Parks. Freshly dissected thyroid glands were weighed on a torsion balance, fixed in Bouin and stained with Heidenhain's hematoxylin and eosin. Küchler (11), using

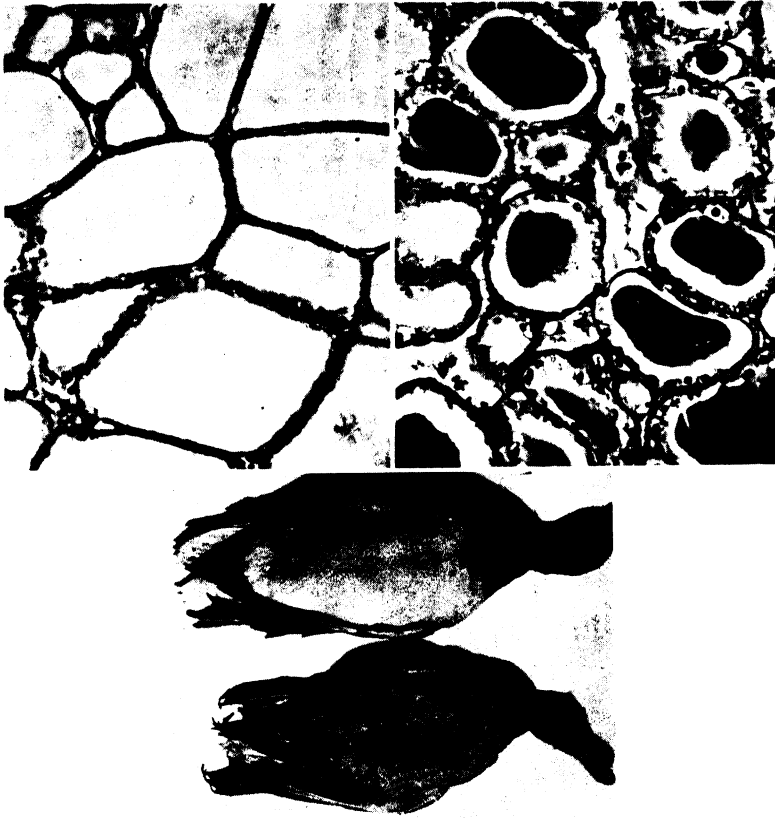


Fig. 1. *Upper left:* Photomicrograph of adult female mallard's thyroid in 'resting,' relatively inactive condition $\times 210$ (Aug. 2, 1944). *Upper right:* Adult female mallard's thyroid in 'active' condition $\times 210$ (Feb. 10, 1946). *Lower:* Ventral views of control male, top, in full breeding plumage; thyroidectomised male, bottom, in breeding plumage on head, neck, upper breast and tail but retained juvenile plumage on abdomen. 'Loose' feathers characteristic of hypothyroidism. Photographed 6 months after thyroidectomy.

this method on avian material, claims that intra-cellular droplets of colloid are shown as colorless vacuoles. In the mallard it was not possible to demonstrate this material by this method nor by that of Dvoskin (12) in glands which by other criteria were highly active. Data on body weight, weight and histology of the gonads, adrenals and thymus of the birds used in the present series have already been reported elsewhere (13, 14).

Results (Table 1 and figs. 2 and 3). The macroscopic anatomy of the mallard's

thyroid conforms to the usual avian pattern of two entirely discrete lobes, one on each side, lying on the carotid artery. Three deviations from the normal arrangement were encountered, all in females. In 2 birds the lobes were connected at their caudal extremities by a thin strand of thyroid tissue running across the trachea. In another female, outside the present series, there was no thyroid on the left, the right lobe being about twice the normal in weight, so that the total amount of thyroid tissue was still about average.

Thyroid Weight. On the whole, glands which were judged active, in histological appearance, were below average in weight; while 'storage' glands were heavy. A complete correlation between weight and activity probably does not appear since a gland will show the picture of a 'storage' phase before colloid storage has caused a marked weight increase. Similarly, early in a secretory phase actual weight loss from

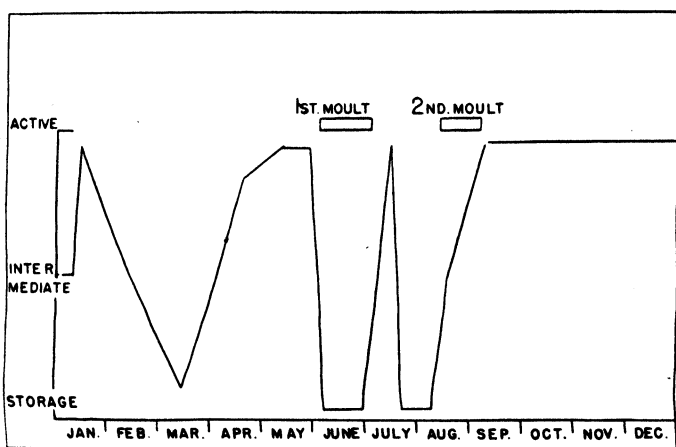


Fig. 2. SEASONAL ACTIVITY of thyroid of adult male mallards (based on histological findings) related to moults.

colloid depletion may be expected to lag behind the appearance of histological activity. Gland weights alone therefore give only a poor indication of the seasonal cycle of activity. The average weight of the thyroid tissue of 28 males was 108.37 mg. and 88.9 mg/kg. body weight. For females absolute average weight was 104.8 mg. and 100.8 mg/kg. body weight. It is doubtful whether this difference in thyroid weight per unit body weight is significant, since owing to unequal numbers and unequal seasonal distribution of the respective data for the two sexes, the figures may be biased.

Histological Appearances. A number of serial sections through the center of each of the two lobes were examined for every bird. For each lobe 10 measurements of vesicle diameter and 'vesicle wall thickness' (depth of tissue between two adjacent vesicles = twice epithelial height + intervening connective tissue \pm capillary, a ready index of epithelial cell height) were made. Vascularity was assessed by the number of non-capillary vessels in 10 high power fields per lobe. Although the histological technique used was unsuitable for cytological detail, two extreme phases of

thyroid activity could easily be recognized on this basis. These may be briefly characterized as follows, quantitative data being based on the above measurements for both thyroids of 10 birds, in each case both sexes being represented. 1) A resting or 'storage' gland with large vesicles average diameter $79.5\ \mu$ with low epithelium and flattened nuclei, average wall thickness $6\ \mu$ and poor vascularity, an average of 8 non-capillary vessels per 10 high power fields. This presumably represents a phase of colloid accumulation but with reduced colloid production and greatly reduced hormone discharge into the circulation. 2) The other extreme labelled 'active' was a phase of maximal secretory activity with increased hormone discharge. Vesicles were small, average diameter $47\ \mu$, epithelium high, average wall thickness $8.5\ \mu$ with rounded, paler nuclei, a well-developed intervesicular capillary network and an apparent increase in larger vessels, average 23 in 10 high power fields. Such glands

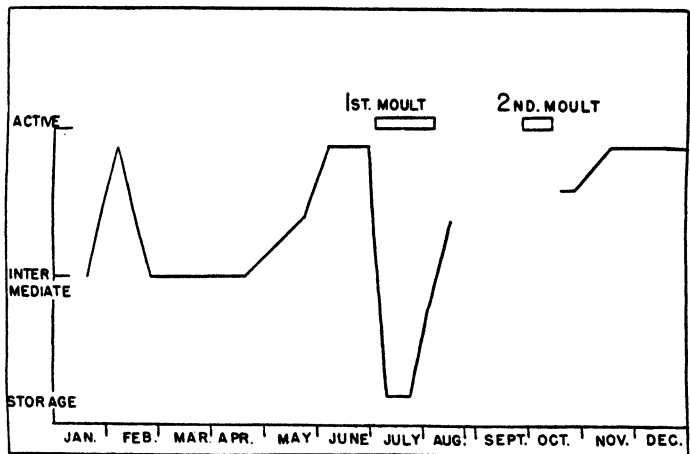


Fig. 3. SEASONAL ACTIVITY of thyroid of adult female mallards (based on histological findings) related to moults.

frequently showed non-staining, approximately circular areas in the peripheral colloid. These also appear in Zenker fixed glands stained with azocarmine and Heidenhain's azan. Since they were always found in 'active' glands, the suggestion of others that they represent a transport phase of the colloid during secretion into the vesicle or during its resorption therefrom appears plausible though their precise nature cannot be regarded as established.

Table 1 summarizes the seasonal phases of gland activity as assessed by these histological criteria in relation to gland weights and plumage; while figures 2 and 3 represent the activity cycle in relation to the moults in graphic form.

Two clearly separated phases of thyroid activity are shown. First, during the colder months, October to January, there is a period of activity in both sexes. A similar increase of thyroid activity during the cooler months has been reported in the house sparrow by Miller (15), who has also induced this response experimentally by exposure to low external temperatures. Undoubtedly this response plays a part in temperature regulation during winter in birds.

Second, a phase of activity is found in the summer which appears to be related to the first moult. In the male, this phase extends from mid-April to late May, preceding the onset of the moult by about one month (fig. 2). In the female, it occurs later, namely in June, but again its onset precedes the moult by about one month (fig. 3). The fact that the summer phase of thyroid activity shows a different seasonal incidence in the two sexes is strong evidence that this activity is in fact correlated with the moult. If any phase of thyroid activity precedes the less severe second moult, it must be of much shorter duration than that preceding the major moult. The active condition of a male thyroid of July 16 may represent this phase. The fact that the phase of thyroid activity precedes rather than synchronizes with the moult is explicable, since an interval between the time of increased release of thyroid hormone and its effect on the rest of the body is to be expected. This expectation is confirmed by Woitkewitsch (6), who showed that in the starling, in which thyroidectomy abolishes the moult, the operation is effective only if it precedes the onset of the moult by a considerable period.

TABLE 2. EFFECT OF THYROIDECTOMY OF 2-MONTH OLD MALLARDS ON BODY WEIGHT AND MOULT
(OPERATION JULY 15-18, 1947)

Males

Operated: 750, 900 gm.¹

Controls: 1050, 1000

1100, 950 gm.

Average: 825 gm.¹

Average: 1025 gm.

Females

Operated: 950 gm.¹

Controls: 1000, 1025, 1000,

1050, 1000, 1150,

950 gm.

Average: 1035 gm.

¹ Body weight when killed, 7 months old (Jan. 6, 1948).

RESULTS OF THYROIDECTOMY

Since Woitkewitsch (6) showed that thyroidectomy performed in ducklings when 5 to 10 days old prevented the moult completely, while on the other hand Chu (10) found that in adult mallards thyroidectomy did not prevent onset of the moult into the eclipse plumage, it was of interest to investigate the effects of thyroidectomy at an intermediate age. A number of mallards were, therefore, thyroidectomized when two months old, July 15 to 18, to observe the effect on the moult from the first juvenile into the first mating plumage due about mid-September. Three birds, 2 males and 1 female, made a complete recovery and survived until January 6, when they were killed. Post-mortem examination of the thyroid region (macroscopic and microscopic) showed that the operation had been complete and no thyroid regeneration had taken place. Table 2 summarizes the effects of the operation on body weight. This shows that the operation delayed growth as indicated by decreased body weight compared to the controls. The effect of thyroidectomy on the moult and formation of the new plumage is shown by the plumage conditions as recorded on November 25 (17 weeks after thyroidectomy of the experimental birds).

Males. Four controls in full breeding plumage, 1 thyroidectomized bird in full breeding plumage, the other showed retention of the juvenile plumage on the abdomen; otherwise full breeding plumage.

Females. Seven controls, all in full breeding plumage, thyroidectomized bird also in full breeding plumage.

The operated birds showed the usual structural feather defects of thyroidectomy. The date of first appearance of the new plumage was variable in the controls of both sexes and no definite lag in appearance of new feathers in the thyroidectomized birds was evident at any time.

Failure of the moult in part of the ventral surface as noted in one of the operated males occurs occasionally in wild mallards wintering in northern latitudes (personal communication of Prof. W. Rowan) and its occurrence in the present instance is, therefore, not necessarily attributable to the thyroidectomy. Hence, it must be concluded that thyroidectomy did not prevent or delay the onset of the moult.

DISCUSSION

The result of 3 thyroidectomy experiments may, by themselves, be regarded as inconclusive, but they are supported by the similar findings of Chu (10). The prevention or postponement of the moult in ducklings operated when under one month old, reported by Woitkewitsch (6), can readily be explained as part of the general retardation of development induced by thyroidectomy, rather than as a specific effect of the operation on the moulting process. The general conclusion that mallards can moult in the absence of the thyroid gland therefore remains unaffected.

Recent observations by Sulman and Perek (17, 18) show that the basal metabolism of hens is raised considerably during the moult. Nevertheless, when this was prevented by administration of thiouracil, the moult was neither postponed nor prevented. The position in the mallard appears to be essentially similar; increased thyroid activity occurs somewhat prior to the moult; by this, the metabolic rate is no doubt raised, presumably in connection with the formation of new feathers. This phenomenon is, however, merely associated with and not a cause of the moult, which still occurs after thyroidectomy. As indicated by the experiments of Chu referred to above, there is, however, a causal relationship between androgenic activity of the testis and the moult in this species.

SUMMARY

Histological observations on 24 adult male and 16 adult female mallards indicate two seasonal phases of increased thyroid activity: *a*) one affecting both sexes from October to January; *b*) one preceding the first, more extensive moult by about one month, occurring in males from April to late May and in females, which moult about one month later, in June. A second, much more transient period of increased thyroid activity may precede the less extensive second moult.

In three birds operated two months before the expected moult from juvenile to first breeding plumage which survived a 6-month period of observation after complete thyroidectomy, the moult was not prevented or postponed, though one bird failed to moult its abdominal feathers.

Three females showed a deviation from the normal macroscopic thyroid pattern, the two lobes being united caudally across the midline of the neck in 2 birds; in another the left thyroid was absent but the right hypertrophied. Mean weights of thyroid tissue were 88.9 mg/kg. body wt. in males and 100.8 mg/kg. body wt. in females.

Thanks are due to the medical research committee of Guy's Hospital Medical School (where the material was prepared) for financial support; to Dr. J. Beattie of the Royal College of Surgeons (England) for facilities to keep ducks at the Buckstone Brown Experimental Farm, and to Sir Arthur Keith and Mr. F. Watson for observations on these birds in the author's absence.

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FLUCTUATIONS OF SERUM CHOLINE IN WOMEN

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SEVERAL lines of evidence suggest that variations in the choline content of serum occur throughout the menstrual cycle. Thus estrogens, which exhibit two maximal levels during the cycle, are known to affect the cholinesterase activity of blood (1-4) and to exert a cholinergic action upon certain tissues (5-8). This subject was recently reviewed comprehensively (9).

PROCEDURES

In order to investigate the above relationship the serum choline content of 36 women was studied. As noted in table 1, there were two categories of subjects, designated *A* and *K* respectively. *Group K* comprised 15 subjects who were patients in the Gynecologic Department of the Municipal Hospital in Copenhagen. The patients selected for study presented insignificant gynecological anamneses and, practically speaking, may be regarded as normal healthy individuals. On each of these subjects a duplicate determination for serum choline content was made only once. *Group A* comprised 21 female medical students with no gynecologic complaints. On most of these subjects several duplicate determinations were made at various times (see table 1, column 3).

In all, 93 duplicate determinations were made. In order to insure uniformity all blood samples were drawn in the morning. In most instances the individuals were fasting when blood samples were drawn. A few by mistake had their breakfast beforehand but this did not seem to have any effect on the choline level of the blood.

Determinations of the choline content in serum were made by acetylation of the serum, whereby the choline was converted to acetylcholine. The method of Abdon and Ljungdahl-Østberg (10), slightly modified, was employed for the acetylation of serum. Blood samples were drawn by venous puncture. The amount of acetylcholine formed was determined by comparing the effect of the acetylcholine obtained by acetylation with the effect of a solution of acetylcholine of known potency on isolated guinea pig intestines (9). The mean of the standard deviation of the individual determinations in duplicate is 9.4 per cent, in single, 12 per cent (9).

RESULTS

Serum Choline. It is readily apparent from table 1 that there was a wide spread of experimental results within the individual days of the cycle and from day to day. It was necessary to investigate whether or not these variations could be attributed, entirely or in part, to variations in the choline content of the diet.

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TABLE I

SUBJECT	DATE	SERUM CHOLINE, MG. %	DAY OF CYCLE	SUBJECT	DATE	SERUM CHOLINE, MG. %	DAY OF CYCLE
<i>Women Students</i>							
A 34	5/ 9/47	0.433	21	A 42	10/ 1/47	0.296	6
A 35	5/28/47	0.368	7		10/ 8/47	0.232	12
	4/28/47	0.242	5		10/23/47	0.728	27
A 37	4/28/47	0.185	26		10/29/47	0.555	5
A 38	5/ 9/47	0.467	4		2/ 3/48	1.210	15
	5/23/47	0.670	18		3/ 2/48	1.370	14
A 40	5/23/47	0.400	1	A 48	3/10/48	2.145	22
	9/25/47	0.425	13		10/ 6/47	0.204	18
	10/ 2/47	0.462	20		10/13/47	0.304	24
	11/17/47	0.304	1		11/ 3/47	0.420	15
	11/24/47	0.433	7	A 53	11/ 3/47	0.442	4
	12/ 8/47	0.433	21		11/10/47	0.104	11
A 41	9/24/47	0.308	6		11/17/47	0.620	18
	10/ 8/47	0.160	20		11/26/47	0.383	27
	10/15/47	0.288	25	A 48	11/ 6/47	0.357	22
	10/22/47	0.447	4		11/20/47	0.672	8
	10/30/47	0.500	12		11/27/47	0.388	15
A 43	9/24/47	0.395	12	A 50	11/ 6/47	0.860	18
	10/ 1/47	0.199	19		11/27/47	0.620	12
	10/ 8/47	0.170	4		12/ 3/47	0.372	17
	10/15/47	0.438	11		4/12/48	0.985	10
	10/22/47	0.402	18	A 51	11/ 5/47	0.343	21
	2/ 2/48	1.375	1		11/19/47	0.440	4
A 44	9/25/47	0.317	25		12/ 2/47	0.565	17
	10/ 2/47	0.379	26		4/29/48	0.605	20
	10/ 9/47	0.398	5	A 52	11/ 5/47	0.240	6
	10/16/47	0.408	12		11/10/47	0.603	20
	10/22/47	0.543	18		12/ 1/47	0.573	6
A 45	9/25/47	0.400	5		12/10/47	0.695	15
	10/ 9/47	0.342	19	A 54	11/ 5/47	0.437	1
	10/16/47	0.328	25		11/19/47	0.570	14
	10/29/47	0.414	10		12/ 3/47	0.380	28
A 46	9/29/47	0.317	2		3/ 2/47	0.847	21
	10/ 6/47	0.317	2	A 55	11/20/47	0.257	5
	10/27/47	0.582	9		11/27/47	0.858	12
	10/29/47	0.550	3		12/ 1/47	0.383	16
A 47	9/29/47	0.762	7	A 57	12/10/47	1.036	13
	10/ 6/47	0.390	15		4/29/47	0.595	21
	10/13/47	0.433	21				
	10/29/47	0.580	4				
<i>Clinic Patients</i>							
K 0	5/21/47	0.422	10	K 26	7/ 8/47	0.169	1
K 13	6/13/47	0.265	17	K 27	7/ 8/47	0.332	6
K 14	6/13/47	0.412	10	K 30	7/10/47	0.253	15
K 15	6/17/47	0.233	7	K 31	7/10/47	0.054	28
K 20	6/24/47	0.278	27	K 32	7/15/47	0.381	15
K 21	6/24/47	0.340	19	K 34	7/15/47	0.305	9
K 22	6/24/47	0.272	26	K 37	7/17/47	0.382	24
K 23	7/ 2/47	0.418	28				

Diet and Serum Choline. Experimenting with animals, Luecke and Pearson (11) found that a dosage of 400 mg. of choline chloride administered daily for 6 days did not raise the choline content of either the liver, kidney or plasma of sheep. Borglin (12) found no indication that the level of choline in human blood changes with the diet. Following the administration of pure choline in very high dosage he observed a sharp rise in choline content followed by a comparatively rapid decline to original levels. The chief purpose of this phase of our study was to ascertain whether or not a diet high in choline or choline-producing substances ingested on the day prior to the drawing of blood samples would influence the serum choline content in the morning of the following day.

For 5 consecutive days analyses of the serum choline content of one patient in the Municipal Hospital in Copenhagen were made. During the first 2 days the patient was given a normal hospital diet. During the following 2 days the patient received 5 gm. of L-methionine, 6 eggs and 130 gm. of protein daily. The results are given in table 2. The patient was a woman (K44, table 2) aged 17. She was admitted to the hospital on January 3, 1948, and was under observation for rheumatic fever. At the time when blood samples were drawn she was non-feverish and without complaints.

It appears from table 2 that the administration of a diet high in choline does not raise the serum choline content of a blood sample drawn the following morning. It is true that relatively great

TABLE 2

DATE	DIET	SODIUM	CHOLINE, MG. PER CENT
1/18/48	8 A.M.	fasting	1.42
1/18/48	7 P.M.	normal	1.40
1/19/48	8 A.M.	fasting	1.24
1/19/48	5 P.M.	normal	1.27
1/20/48	8 A.M.	fasting	0.90
1/20/48	6 P.M.	spec. diet	2.13
1/21/48	8 A.M.	fasting	0.81
1/21/48	4 P.M.	spec. diet	0.98
1/22/48	8 A.M.	fasting	1.70

variations are observed, but these variations do not seem to be attributable to the diet. The patient began to menstruate on January 19, 1948. It is possible that the variations in serum choline correspond to some change associated with the menstrual cycle. It is difficult, however, to offer an explanation for the single high finding of 2.13 mg. per cent.

A comparison of these data with the results reported by Borglin would seem to justify the assumption that a normal diet does not cause any appreciable variation in the serum choline content. In any case, experimental conditions must be said to be uniform if all blood samples are drawn in the morning from fasting individuals. The diet ingested on the previous day, even though very high in choline, should not have any effect on the serum choline content. Since the mean of the standard deviation of the individual determinations in duplicate summarized in table 1 is only 9.4 per cent, and since the diet does not have an effect on the serum choline content, other factors must be responsible for the variation in the values presented in table 1.

Menstrual Cycle and Serum Choline. When the serum choline concentrations given in table 1 are plotted as a function of the menstrual cycle, using fluctuating means of the values obtained over a period of 5 days, the stippled curve in figure 1 is obtained. The curve was constructed on the basis of fluctuating means of the values obtained over a 5-day period in order to eliminate the errors which may arise when fixing the day of the cycle. The cycle is here assumed to be 28 days long. It will be seen from the curve in figure 1 that maximum values were encountered on the 14th day, minimum values on the 26th day.

A statistical computation of the significance of the difference in serum choline concentration between the 26th and the 14th day shows that the probability of such a great difference between the two means being a chance occurrence is 1.4 ($P = 1.4\%$). This means that the difference in choline concentration between the 26th and the 14th day is significant. The significance of the difference in choline concentration between the 22nd and the 26th day of the cycle (P) is found to equal 8.4 per cent. Thus the difference is probably significant. The decrease in serum choline concentration is not significant since P equals 14.7 per cent.

In summary, therefore, decrease in choline concentration of the serum occurring between the 14th and the 26th day of the menstrual cycle has statistical significance,

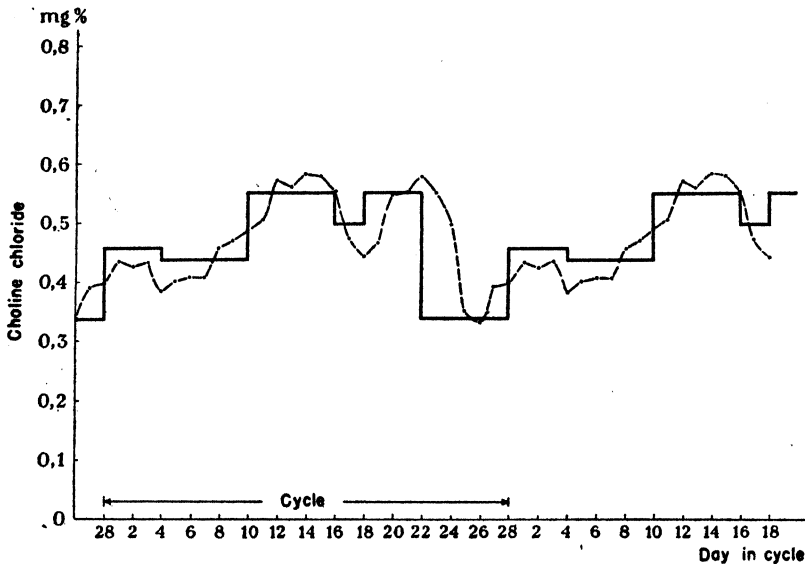


Fig. 1. CURVES indicate the serum choline content in mg. % corresponding to the different days within the menstrual cycle. Data obtained in 93 duplicate determinations. The stippled curve was plotted on the basis of fluctuating means of values obtained over a 5-day period. The solid line indicates the mean at different intervals.

whereas there is little statistical probability that the drop does not occur before the 22nd day. Accordingly, one may say that a relationship exists between the choline level of the serum and two particular phases of the menstrual cycle. The fact that a relationship is established statistically between the serum choline content and the menstrual cycle does not offer an explanation for the great variations occurring both from day to day and within the individual days of the cycle (table 1). This variability is in part attributable to the fact that conditions other than hormonal ones affect the determinations which are reported above. Among these are seasonal climatic factors.

Seasonal Variations of Serum Choline. In the course of further experiments it appeared that the serum choline content is subject to marked variation from season to season. The cyclic variation with the menstrual cycle is superimposed upon that of this basic annual cycle. The seasonal variation is

shown in figure 2. The height of the columns indicates the mean of the total number of choline values measured during the month. The total number of duplicate determinations made during each month is given at the bottom of the columns. No measurements are available, however, for the month of August, 1947.

It will be seen that the monthly mean levels of choline were found to be highest in the months of February and March and lowest in the months of June and July. A statistical computation of the difference between maximum and minimum values—even though based on the data of women only (13 women in July and 3 women in March)—reveals that P equals less than one per thousand, which means that the difference is highly significant.

The curve in figure 1, representing the relation of variations in choline content to phases of the menstrual cycle, was constructed on the basis of values obtained over a 12-month period. Eight determinations were made on menstruating women during the months of January, February, March and April, 1948, whereas 85 measurements were made during the remainder of the year. Accordingly, an attempt has been made to determine statistically whether or not the maximum serum choline

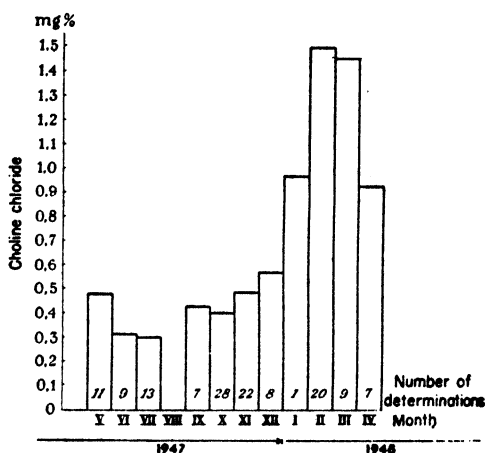


Fig. 2. SEASONAL VARIATIONS in the choline content of the serum.

level occurs about the 14th day irrespective of the season. This is so, since the value of P in each case equals 1 per cent, which means that the difference in serum choline levels between the 14th and the 26th day is still significant.

From this it may safely be concluded that a relationship clearly exists between the concentration of serum choline in the average woman and the day of her menstrual cycle. The average serum choline concentration is highest on the 14th day and lowest on the 26th day of the cycle.

DISCUSSION

A cyclic variation in the menstrual cycle of the choline level of the serum would appear to be correlated with corresponding hormonal changes. If so, the existence of a relationship between estrogen and choline may exist. From the following it is clear that such a correlation does hold, for serum estrogen and choline content of the serum vary together during the menstrual cycle.

Fluhmann (13) studied the estrogen content in the serum of 80 young women. It appears from his investigations that the estrogen content is highest around the 14th day and lowest around the 27th day of the menstrual cycle. Markee and Berg (14) conducted similar experiments on 75 young women. The estrogen curve given by

them shows the highest values grouped around the 14th-15th day and the lowest values around the 26th to 28th day.

In summarizing these results it seems reasonable to deduce that the concentrations of both estrogen and choline vary during the menstrual cycle in such a manner that there is a coincidence of the maxima and the minima of the two curves. This would suggest a possible relationship between estrogen and choline. The established effect of estrogen upon serum cholinesterase in rats, rabbits and guinea pigs may provide the basis for this relationship (1-4).

SUMMARY

Serum choline concentration is related to the menstrual cycle with the highest value around the 14th day and the lowest around the 26th. A possible relationship between choline and estrogen is pointed out. The choline content of the serum is on the average about 5 times higher in the month of February and March than in the months of June and July. (The experiments were carried out in Denmark in the period, May, 1947-May, 1948.)

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GLYCOGENIC EFFECT OF ADRENAL CORTICAL EXTRACT

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KELLEY and McDonald (1) reported alteration of the glucose tolerance of dogs exposed to simulated altitudes of 24,000 feet. During an attempt to find an explanation for this, we found that adrenal cortical extract caused a marked increase in the ability of dogs to form glycogen from glucose. A full report offering an explanation of the altered glucose tolerance is being presented (2) but it was felt that the importance of this glycogenic property of adrenal cortical extract merited its appearance in a separate paper.

METHODS

The animals used were 6 well-trained Dalmatian coach hounds, 4 of which were litter mates. The dogs were loosely restrained in a supine position on animal boards, no anesthesia being used. Blood samples were obtained by femoral arterial puncture or by external jugular vein puncture. The dogs were fasted for 12 hours prior to the test. In experiments at a simulated altitude of 24,000 feet, the dogs were restrained prior to decompression and then decompressed at the rate equivalent to an ascent of 2000 feet per minute. This, plus previous experience of the dogs in the decompression chambers, we hoped would minimize stimulation of the sympathico-adrenal system.

The glucose tolerance test consisted of injecting intravenously 0.5 gm. of glucose/kg. of body weight, and drawing blood samples immediately before, 15 and 30 minutes, 1, 2 and 3 hours after the injection of the glucose. All blood sugar determinations were made by the method of Horvath and Knehr (3). The blood lactic acid was determined by the method of Barker and Summerson (4) and the blood pyruvic acid by the method of Friedemann and Haugen (5).

RESULTS

The data on which this report is based are listed in table 1. The conditions under which these data were obtained, as indicated in the table headings, were during the course of standard intravenous glucose tolerance tests: *a*) at ground level (750 feet above sea level), *b*) at decompression equivalent to an altitude of 24,000 feet and beginning 1 hour after the intraperitoneal injection of 2 cc/kg. of body weight of commercial adrenal cortical extract, and *c*) at ground level at varying intervals after similar administration of the extract. Control experiments were performed giving the adrenal cortical extract without the glucose, *d*) at simulated 24,000 feet, and *e*) at ground level.

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TABLE I

CONDITIONS	DOG NO. ¹	BLOOD											
		Glucose, mg/100 cc.						Lactic Acid, mg/100 cc.					
		B ₁	B ₂	Hours After B ₂				B ₁	B ₂	Hours After B ₂			
				¼	½	1	2 3			¼	½	1	2 3
1. Standard GTT (intravenous)	1V		98	163	130	113	98		15.0	18.2	21.8	16.0	15.8
	2V		102	157	126	118	93		24.0	17.5	17.2	12.0	11.3
	3V		132	200	182	134	132		37.3	22.1	13.7	17.0	20.6
	4V		118				215		14.9	13.4	19.4	13.4	18.5
2. GTT with ACE (24,000 ft.)	2AE	88	121	124	147	161	153	127	9.2	17.5	16.9	16.4	7.3
	3AE	96	86	84	110	66	78	70	5.0	23.0	21.8	16.3	7.8
	4AE	116	98	78	122	102	152	148	8.6	27.8	21.7	28.9	32.0
	2AU	91	71	139	124	122	122	122	13.6	19.2	22.4	24.0	14.0
	3AU	88	77	115	91	91	96	91	6.60	19.2	17.5	13.4	22.7
3. GTT with ACE, ground level	4AU ²	88	75	91	80	84	96	109	10.4	23.0	17.2	14.0	42.0
	1VE	105	225	150	147	143	143	143	18.6	14.5	10.4	12.2	32.0
	2VE	89	143	135	137	135	131	128	15.1	13.4	10.9	13.3	36.6
	2AE ²	102	184	152	127	127	113						
4. ACE alone at altitude	6AU	89	122	164	117	100	135	131					
	5AU	96	105	164	135	122	117	124					
	5AE	70	66	76	76	72	72	6.2	13.8	10.3			
	6AE	66	82				104	8.4	18.6				
5. ACE alone, ground level	2AU		96	91	91				7.80	7.14	13.6		
	3AU		91	91	88				5.16	7.08	6.60		
	4AU		89	89	88				6.20	9.54	10.4		
	5AU		96	96	109	105							
	6AU		89	88	84	122							

B₁—In altitude studies, the baseline before decompression. In all other studies, the baseline before any medication was given.B₂—Sample at the beginning of the actual or simulated Glucose Tolerance Test. GTT—Glucose Tolerance Test. ACE—Adrenal Cortical Extract.¹ Dog received only part of glucose to be injected. ² V or A designates the sample being taken from the vein or the artery. U or E designates the adrenal cortical extract as being U—Upjohn's Adrenal Cortex Extract, or E—Eschatin (Parke, Davis and Company). : Cortical extract and glucose were injected at the same time in this animal.

Administration of 0.5 gm. of glucose/kg. of body weight to 4 of the dogs produced a minimum increment in the blood sugar of 55 mg/100 cc. at the 30-minute period. All levels returned to the baseline at the end of 3 hours. The blood lactic acid and pyruvic acid values in this test, unfortunately, were done on venous samples which later were found to be greatly affected by such minor disturbances as excitement and stasis (2). However, there were no marked increases in either throughout the test.

Administered glucose under the influence of adrenal cortical extract produced rather startling results. In the studies using the extract at a simulated altitude of 24,000 feet, the act of decompression itself produced no uniform change in the blood glucose level but, in the case of Eschatin², the administered glucose disappeared from the blood stream within 15 minutes. In the case of Upjohn's Adrenal Cortex Extract, one dog showed rapid disappearance of the glucose while it was not markedly evident in the other one. However, in this dog, it should be noted that the blood lactic acid and pyruvic acid were greatly elevated after injection of the glucose and continued so generally throughout the test.

The added glucose at ground level also disappeared from the blood stream rapidly under the influence of adrenal cortical extract. In the case of the first two dogs, Eschatin was given only 15 minutes before the glucose. The extract itself raised the blood glucose level markedly, but the added glucose had completely disappeared from the blood stream at the end of 30 minutes. The experiment on the second dog was then repeated giving the extract and the glucose at the same time and the 15-minute increment was only 82 mg. per cent. In the case of Upjohn's Adrenal Cortex Extract, all of the injected glucose was cleared from the blood within 30 minutes in the first of two dogs and the second showed a 30-minute increment lower than expected in a normal glucose tolerance test. Urine collection for the first hour of the test in these two dogs showed reducing substances less than that equivalent to 1 gm. of glucose when titrated with Benedict's solution.

In the control studies, Eschatin was given one hour previous to decompression to a simulated altitude of 24,000 feet and very little effect on the blood glucose was evident. As can be seen from the previous experiments, Eschatin caused a rise in blood glucose 15 minutes after injection. This was quite high and presumably due to presence of some adrenalin as the glucose value at one hour was usually back to normal. Therefore, Upjohn's Adrenal Cortex Extract, which reputedly contains negligible adrenalin, was tried to ascertain its early effect. In five determinations there was no alteration of blood glucose at the 15-minute level although sometimes there was elevation at one hour.

DISCUSSION

In a standard glucose tolerance test, the rapidity with which the glucose disappears from the bloodstream is determined for the most part by how rapidly glycogen is formed. The formation of fat is a much slower process and injecting carbohydrate increases oxidative processes commensurate only with the increase theoretically expected from the specific dynamic action of glucose (6). Since it has been shown that

² Eschatin is an adrenal cortical extract produced by Parke, Davis and Company.

adrenal cortical extract actually promotes the conversion of both fat and protein to carbohydrate metabolites (7) and that injection of adrenal cortical extract in the hepatectomized rat causes no influence on the blood sugar (8), it follows that the rapid clearance of added glucose after the injection of adrenal cortical extract occurred because the animals had an increased ability to form glycogen.

It has been known for many years that adrenalectomy makes it difficult to maintain normal glycogen stores (9-13) particularly in the liver, unless salt and water balance are maintained carefully and the animals are well-fed (14). However, administration of adrenal cortical extract enables an adrenalectomized animal to maintain perfectly adequate glycogen stores (14, 15). Even further, giving adrenal cortical extract to normal animals causes increase to above normal glycogen deposits (16, 9). Moreover, Sundstroem and Michaels (17) found that rats kept at simulated high altitudes were able to maintain amazingly high liver glycogen levels in comparison to the nutritional state of the rest of the body as a whole. Also, the higher the altitude, the higher the glycogen content, presumably corresponding to the amount of hormones produced by the adrenal cortex. From what can be gathered from literature, many assume that all this glycogen deposition is secondary to gluconeogenesis (18). Several reports have come out, however, to support the premise that there is actually a positive effect on glycogen deposition by adrenal cortical extract. Britton and his workers actually proposed this many years ago (9), and it has been suggested as a means of assay of adrenal cortex preparations (9, 19). They however, at that time apparently did not convince everyone that adrenal cortical extract actually had glycogenic powers; but in 1940, Corey and Britton (20) perfused livers and found that adrenal cortical extract did enhance glycogen deposition in these livers. In addition to this, a preliminary report by Chiung-yun Chin and Needham (21) reported that adrenal cortical extract enhanced glycogen deposition in tissue slices from added glucose, sodium pyruvate, or DL-alanine.

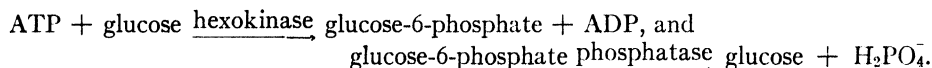
If adrenal cortical extract actually enhances glycogen deposition from glucose, then where in the metabolic cycle could it act? Glycogen deposition might be enhanced by *a*) rapid removal of inorganic phosphate, *b*) more rapid transfer of glucose through the cell membrane, *c*) any reaction which would increase the concentration of glucose-6-phosphate, or *d*) an increase in the turnover of the enzymes concerned in the reversible reactions converting glucose-6-phosphate to glycogen.

(*a*) Kalckar (23) estimates the penetration of inorganic phosphate in muscle to be approximately 1 γ of P per minute per gram of muscle. He further gives evidence to support the premise that phosphate penetrates the cell boundary by a purely physical means. If, then, the transfer of the organic phosphate in liver cells were of the same order, the simple transfer of inorganic phosphate from the cell would be consistent with the rate of disappearance of glucose in a standard glucose tolerance test. However, inorganic phosphate must continually be used to rejuvenate the adenosine triphosphate used in the formation of glucose-6-phosphate from glucose, and Kalckar estimated the rate at which adenosine triphosphate can be rejuvenated in muscle cells to be in the order of 20 to 30 γ of P per minute per gram of muscle, and in liver 15 γ of P per gram of liver per minute. This provides a means for removal of phosphate much greater than is called for in a standard glucose tolerance test, so

it is unlikely that the removal of inorganic phosphate is the limiting factor determining the rapidity with which an organism can form glycogen.

(b) The transfer of glucose across the cell membrane need not be considered the limiting factor because a man doing hard physical labor may use energy to the equivalent of 100 grams of glucose per hour, which after his existing glycogen stores are depleted will be supplied largely in the form of glucose to the muscle cells. This is far above the rate needed in a normal glucose tolerance test.

(c) The concentration of glucose-6-phosphate may be increased in several ways. Since pyruvic acid concentration increases in these experiments, it would not be inhibition of any of the reversible reactions between glucose-6-phosphate and pyruvic acid; therefore, it would be 1) increase in the concentration of adenosine triphosphate, 2) decrease in the turnover of the phosphatase enzyme converting glucose-6-phosphate to glucose, or 3) increase in the turnover of hexokinase converting glucose to glucose-6-phosphate. All these possibilities can be excluded as they would all result in a decrease in blood sugar when the adrenal cortical extract was first given. In other words, the glucose-6-phosphate and glucose are at equilibrium with each other because of the equalization of the two reactions:



That this is so can be shown by giving insulin which allows a faster turnover of hexokinase (22) and a resultant fall in the blood glucose. Further, it has been shown that, although insulin may enhance glycogen deposition in normal animals with normal or low blood sugars, it enhances glycogen deposition only slightly when glucose is given to raise the blood sugar level (24).

(d) We are left, then, with the possibility of an increase in turnover of enzymes in the reversible reactions converting glucose-6-phosphate to glycogen. The end equilibrium of this system as expressed by its equilibrium constants is not changed, but the speed with which equilibrium is reached is altered.

With this in mind, consider the case where Upjohn's Cortex Extract, containing negligible amounts of adrenalin, was given alone. There was no appreciable effect on the blood glucose in the first 15 minutes. The increase in some instances by one hour would be due to the well-known gluconeogenic effect (25) of adrenal cortical extract, which is maximal about 4 hours after injection (26). Also, when adrenal cortical extract was given and the dogs were subjected to a simulated altitude of 24,000 feet, there was enough oxygen present to maintain the aerobic metabolism approximately as well as at ground level (27). So again the equilibrium was not upset, and the blood glucose was not altered appreciably until gluconeogenesis was active. On the other hand, when glucose was given intravenously before gluconeogenesis (due to the adrenal cortical extract) had been increased to a large degree, the excess glucose upset the equilibrium but disappeared from the blood stream at a rate much above that in a normal animal. The ability of the system to utilize glucose and re-establish equilibrium had increased.

An increase in turnover of the enzymes converting glucose-6-phosphate to glycogen is entirely consistent with the observation (28, 12) that the response of adrenalectomized animals to injected adrenalin is much reduced even though their glycogen

stores are fairly high. It has further been shown that hypophysectomized animals, after about 30 days, show no hyperglycemic response to injected adrenalin even with adequate glycogen stores (29, 30). In this case, it could be possible that the adrenal cortex undergoes secondary degeneration with a reduced amount of cortical hormone resulting in a decreased turnover of the enzymes in question so that no marked hyperglycemic response occurs from the adrenalin.

Using Cori's work on the hexokinase reaction (22), together with the effect of adrenal cortical extract on the conversion of glucose-6-phosphate to glycogen, one can easily explain the glucose tolerance of a person with Addison's disease. The intravenous glucose tolerance test of a patient with Addison's disease (31) typically shows a lower than normal peak after injection, but a greatly prolonged return to the original blood glucose level. As there are reduced amounts of adrenal cortical hormone present, the hexokinase is not inhibited so there is a rapid uptake of glucose early in the test with the formation of increased amounts of phosphorylated metabolites, resulting in a low increment due to the injected glucose. Then, since the conversion of glucose-6-phosphate to glycogen is not as rapid as when adrenal cortical hormone is present in larger amounts, it takes longer than normally to deposit glycogen and reach the original blood level.

The reaction of *dog* 2AU in the test done with adrenal cortical extract at 24,000 feet can now be explained further too. It will be noted that, early in the test, the lactic acid was increased more than that of the other dogs indicating gluconeogenesis had begun earlier and thus helped maintain the blood glucose at a higher level.

Colowick and Sutherland (32), using purified enzymes, found that the reaction *in vitro*:

glucose-6-phosphate $\xrightarrow{\text{phosphoglucomutase}}$ glucose-1-phosphate
was not as rapid as:

glucose-1-phosphate $\xrightarrow{\text{phosphorylase}}$ glycogen — H_2PO_4^- .

The active constituents of adrenal cortical extracts are generally accepted to be steroids; so the outcome of further work probably will be that these steroids, or a steroid, acts to activate phosphoglucomutase. An additional possibility would be a secondary change in concentration of some other factor such as magnesium ion.

SUMMARY AND CONCLUSIONS

Dogs previously given 2 cc/kg. of body weight of commercial adrenal cortical extract intraperitoneally were found to be able to clear their blood of intravenously injected glucose much more rapidly than without the extract. At this same time, the blood lactic acid and pyruvic acid values were elevated. Giving adrenal cortical extract or glucose in normal animals has not been shown to increase the basal metabolic rate, so the glucose must have been transformed to glycogen. Since it is known that liver glycogen stores are increased when adrenal cortical hormones are increased and that it is difficult to maintain glycogen stores when they are absent, it follows that adrenal cortical extract speeds the formation of glycogen. This must occur by increasing the turnover of the enzymes in either the reaction:

glucose-6-phosphate phosphoglucomutase glucose-1-phosphate

or

glucose-1-phosphate phosphorylase glycogen + H_2PO_4^- .

The most probable mechanism is that an adrenal cortical hormone acts as an activator to phosphoglucomutase.

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GLUCOSE TOLERANCE OF DOGS AS ALTERED BY ATMOSPHERIC DECOMPRESSION

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IN A previous publication (1) it was reported that unacclimatized dogs exhibit a marked decrease in tolerance to glucose administered intravenously during acute exposure to simulated altitudes. Work has continued in an attempt to elucidate the mechanisms responsible for this phenomenon.

When attention is given to the factors responsible for determining the existing blood glucose level of an animal, the picture is seen to be extremely complex. A great number of factors come into play as there is a continuous state of flux among the great number of substances of the metabolic pool. There is constant interplay among not only substances labelled carbohydrate intermediates, but also with proteins and fats. The blood glucose level is determined by the sum total of all these reactions which are, in turn, influenced by many things; such as, concentration of the various substrates, oxygen tension, concentration of enzymes and co-enzymes, and by hormones. The adrenal hormones and insulin have a particularly marked and immediate effect. Considering the marked alteration of adrenal activity in the alarm reaction as proposed by Selye (2), it appears that the three factors likely to exert the predominant effects in altering glucose tolerance at altitude are *a*) the effect of low oxygen tension upon the activity of the various enzymes and the equilibria of these enzymatic systems, *b*) increased activity of the sympathico-adrenal system, and *c*) increased adrenal cortical activity. Therefore, attack of the problem consisted of an attempt to evaluate the relative importance of these three factors upon glucose tolerance.

The position of pyruvic acid and lactic acid in anaerobic glycolysis as expressed by the Embden-Meyerhof scheme has been well established. Further, the importance of pyruvic acid situated at the crossroads, so to speak, of intermediary metabolism, as an intermediary metabolite of carbohydrate, fat and protein is recognized (8). Therefore, it was reasoned that additional information could be obtained from the determination of blood lactic and pyruvic acid during the experiments.

METHODS

The animals used in these experiments were 6 well-fed, well-trained Dalmatian coach hounds, 4 of which were litter mates. The dogs were loosely restrained in a supine position on animal boards. No anesthesia was used and blood samples were at first taken by external jugular vein puncture, but later by femoral arterial puncture. The dogs were fasted 12 hours prior to experiments. In all experiments conducted with the animals subjected to reduced pressure, a standard procedure was adhered to under which the dogs were restrained on animal boards in the low-pressure chamber and decompression to a simulated altitude of 24,000 feet was accomplished at a rate equivalent to an ascent of 2000 feet per minute. Within 5 minutes after the desired decompression was reached, the fasting blood samples were drawn. In order

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to minimize the acclimatization factor, an interval of at least one week was interposed between successive decompressions.

For the intravenous glucose tolerance test, a test dose of 0.5 gm. of glucose/kg. of body weight was given. Blood samples were drawn immediately before the glucose was given, sometimes 15 minutes after injection, and in all cases at 30, 60, 120 and 180 minutes after injection of the glucose. All blood sugar determinations were made by the method of Horvath and Knehr (3).

Blood lactic acid was determined by the method of Barker and Summerson (4) and the pyruvic acid by the method of Friedemann and Haugen (5). The precautions recommended by Friedemann and Haugen were carefully followed in the obtaining and subsequent handling of the blood samples. In the studies made in the low-pressure chamber, a portable ice container was provided so that syringes might be cold at time of use, and the precipitated samples kept refrigerated until they were analyzed.

In the experiments in which adrenalin was given, it was administered by constant intravenous drip at a rate of 40 gamma per minute throughout the entire glucose tolerance test.

RESULTS

The experimental results obtained with regard to blood glucose, lactic acid and pyruvic acid levels, and the lactic acid-pyruvic acid relations under various conditions are shown in table 1. The conditions under which the data were obtained are indicated in the table headings together with the experiment number. A standard glucose tolerance test was made *a*) at ground level (750 ft. above sea level), *b*) at the equivalent of 24,000 ft. altitude, *c*) at ground level during a constant infusion of adrenalin, *d*) at ground level begun 15 minutes after cessation of 3 hours of a constant infusion of adrenalin, *e*) at the equivalent of 24,000 ft. altitude beginning one hour after the intraperitoneal injection of 2 cc/kg. of body weight of adrenal cortical extract, *f*) at ground level after the injection of the same amount of extract intraperitoneally, the last two receiving their cortical extract one hour before beginning the test, the first two beginning the test 15 minutes after, and the third immediately after the cortical extract was injected, and *g*) beginning immediately after a 3-hour exposure to simulated 24,000 feet. The remainder of the experiments were simulated tests where glucose was not injected to show the response of the animal, *h*) to altitude (24,000 feet) alone, *i*) to a continuous infusion of adrenalin at ground level, *j*) to adrenal cortical extract alone at altitude (24,000 feet), and *k*) Upjohn's Adrenal Cortex Extract alone at ground level.

Intravenous Glucose Studies. The response of the dogs to the stress of intravenously administered glucose showed that (*experiment 1*), during the ordinary glucose tolerance test, the 30-minute level of blood glucose was at least 55 mg. above the baseline in all cases and that values had returned to the baseline by 3 hours. Lactic and pyruvic acid levels showed no significant change.

The response to glucose (*experiment 6*), at altitude, in no case showed a 30-minute increment in blood glucose as high as the minimum increment for the test

TABLE I

CONDITIONS	DOG ¹ NO.	BLOOD													
		Glucose, mg/100 cc.							Lactic Acid, mg/100 cc.						
		B ₁	B ₂	Hours After B ₂					B ₁	B ₂	Hours after B ₂				
				¼	½	1	2	3			¼	½	1	2	3
1. Standard GTT (in- travenous)	1V		98		163	130	113	98		15.0		18.2	21.8	16.0	15.8
	2V		102		157	126	118	93		24.0		17.5	17.2	12.0	11.3
	3V		132		200	182	134	132		37.3		22.1	13.7	17.0	20.6
	4V		118			215	162	123		14.9		13.4	19.4	13.4	18.5
2. GTT with ACE (24,000 ft.)	2AE	88	121	124	147	161	153	127	9.2	9.6	17.5	16.9	16.4		7.3
	3AE	96	86	84	110	66	78	70	5.0	6.0	23.0	21.8	16.3		7.8
	4AE	116	98	78	122	102	152	148	8.6		27.8	21.7	28.9		32.0
	2AU	91	71	139	124	122	122	122	13.6	15.8	19.2	22.4	24.0	14.0	11.8
	3AU	88	77	115	91	91	96	91	6.60	9.84	19.2	17.5	13.4	22.7	24.4
	4AU ²	88	75	91	80	84	96	109	10.4	10.4	23.0	17.2	14.0	42.0	35.5
3. GTT with ACE ground level	1VE	105	225		150	147	143	143	18.6	18.8		14.5	10.4	12.2	32.0
	2VE	89	143		135	137	135	131	12.8	15.1		13.4	10.9	13.3	36.6
	2AE ³		102	184	152	127	127	113							
	6AU	89	122	164	117	100	135	131							
	5AU	96	105	164	135	122	117	124							
4. ACE alone at alti- tude	5AE	70	66		76			72	6.2	13.8		10.3			14.4
	6AE	66	82		76			104	8.4	18.6					19.2
5. ACE alone, ground level	2AU		96		91		91			7.80	7.14		13.6		
	3AU		91		91		88			5.16	7.08		6.60		
	4AU		89		89		88			6.20	9.54		10.4		
	5AU		96		96	109	105								
	6AU		89		88	84	122								
6. Altitude GTT (24,000 ft.)	1V	82.1	89		121	156	150	140	23.7	40.8		49.2	81.0	70.2	74.7
	2V		63		113	92	98	117		30.0		54.0	85.0	66.6	54.0
	3V		76		117	93	87	105		39.3		33.9	52.2	63.3	78.6
	4V		76		100	85	76	82		26.5		49.3	44.9	98.2	80.4
7. GTT under adrena- lin	1V		180		206	210	200	193		72.2		96.5	82.6	115.4	93.6
	2V		225		225	235	195	212		67.9		98.9	115.7	76.8	70.6
	3V	85	122		180	163	159	135	43.4	51.6		66.3	93.2	70.1	60.6
	4V	100	105		173	163	152	163	90.6	117.8		114.9	132.9	112.2	57.3
	3A	102	147	180	200	173	185	163	4.50	13.1	18.6	14.0	9.2	14.6	20.6
	4A	63	117	210	210	173	163	173	3.96	11.5	20.3	21.2	24.8	28.6	31.1
8. GTT after adrena- lin	4V	75	91		131	102	90	100	8.6	7.6		7.4	7.2	7.6	6.2
	3V	68	102		131	105	96	89	7.2	7.2		7.6	6.1	3.7	7.2
	2A	147	147	200	173	152	127	102	48.8	45.5	41.2	33.5	15.8	2.40	1.26
	6A	193	159	200	210	152	113	122	50.9	50.9	34.1	14.6	9.2	2.40	1.80
9. GTT after altitude	3V		77		127	113	109	77		16.0		21.7	18.2	10.8	10.0
	4V		68		122	122	105	80		20.2		11.0	12.1	12.4	10.9
10. Altitude alone (24,000 ft.)	1V		64		75	93	96	96	96.3			140.9	163.8	131.6	122.9
	2V		75		82	91	104	115	46.4			99.9	99.5	108.9	90.0
	3V		50		80	97	127	127	15.8			32.6	49.6	70.8	72.0
	4V		59		59	59	84	89	31.4			53.2	84.8	74.8	76.0
	1A		61		61		80 ⁴		8.4			8.1		8.5 ⁴	
	2A		77		91		102 ⁴		7.4			5.5		7.8 ⁴	
	3A		104		96		75 ⁴		7.4			11.6		16.9 ⁴	
	4A		75		100		104 ⁴		11.6			12.2		17.5 ⁴	
	5A		59		77		68 ⁴		16.2			20.6		21.2 ⁴	
	6A		131		100		104 ⁴		5.4			19.2		24.0 ⁴	
11. Adrenalin alone ¹	1V	104	120		111	109	100	100	15.7	19.6		17.2	13.3	13.4	11.0
	2V	100	109		113	111	118	127	20.0			14.6	10.0	12.4	11.0
	3V		117		100	117	114	88		19.3		23.3	18.1	39.6	47.2
	4V		93		112	114	145	116		42.1		30.0	26.9	25.3	28.0

B₁—In altitude studies, the baseline before decompression. In No. 8, the sample immediately on cessation of the adrenalin infusion. In all other studies, the baseline before any medication was given.

B₂—Sample at the beginning of the actual or simulated Glucose Tolerance Test. GTT—Glucose Tolerance Test. ACE—Adrenal Cortical Extract.

TABLE I (Continued)

CONDITIONS	DOG ¹ NO.	BLOOD															
		Pyruvic Acid, mg/100 cc.						Lactate-Pyruvate Ratio									
		B ₁	B ₂	Hours After B ₂					B ₁	B ₂	Hours After B ₂						
				½	½	1	2	3			½	½	1	2	3		
1. Standard GTT (intra-venous)	1V 2V 3V 4V		0.82 1.08 0.74 0.98			1.12 0.88 0.84 0.80	1.30 1.03 0.52 0.53	1.04 0.60 0.50 0.48	1.04 0.64 0.66 0.80		18.3 22.2 50.5 15.2			16.3 19.9 26.3 16.8	16.7 16.7 26.3 36.6	15.4 20.0 34.0 28.0	15.2 17.7 31.2 23.2
2. GTT with ACE (24,000 ft.)	2AE 3AE 4AE 2AU 3AU 4AU ²	0.88 0.58 0.96 0.71 0.40 0.53	0.70 0.70 0.84 0.87 0.80 0.78	1.28 1.44 1.54 1.12 1.10 1.12	1.22 1.74 1.56 1.17 1.25 1.19	1.04 1.06 2.16 2.11 1.45 1.52		0.90 0.78 1.90 1.82 1.97 2.56	0.90 0.78 9.0 19.2 16.5 2.50	13.7 8.6 18.0 18.2 12.3 13.3	13.7 16.0 18.0 17.1 17.4 20.5			13.8 12.5 13.9 19.1 14.0 14.5	15.8 15.4 13.4 11.1 9.2 9.2		8.1 10.0 16.8 6.5 10.0 14.2
3. GTT with ACE ground level	1VE 2VE 2AE ³ 6AU 5AU	1.54 1.12	1.00 1.46			0.94 0.94	1.16 0.66	0.58 0.70	1.30 2.34	12.1 11.4	18.8 10.3			15.4 14.3	9.0 16.5	20.7 18.6	24.6 15.6
4. ACE alone at altitude	5AE 6AE	0.90 0.68	0.70 1.42			0.84 1.52			1.28 1.68	6.7 12.3	19.7 13.1			12.3			11.3 11.4
5. ACE alone, ground level	2AU 3AU 4AU 5AU 6AU		0.97 1.13 1.14	0.46 0.51 0.68		0.71 0.40 0.53					8.0 4.6 5.5	15.5 13.9 14.0			19.2 16.5 19.6		
6. Altitude GTT (24,000 ft.)	1V 2V 3V 4V	1.25	1.20 2.70 2.60 1.10			2.44 2.56 2.70 1.98	2.48 3.84 2.34 2.48	2.07 2.78 2.76 2.54	2.47 2.74 3.46 3.58	19.0 11.1 15.1 24.1	31.6 11.1 15.1 24.1			20.2 21.1 12.6 24.9	32.6 22.2 22.3 18.1	33.8 24.0 22.9 38.6	30.1 19.7 22.7 22.4
7. GTT under adrenalin	1V 2V 3V 4V 3A 4A		2.28 1.44 0.62 0.58 0.90 1.52			3.88 3.84 1.44 2.30 2.06 3.42	3.82 4.88 1.62 2.22 2.22 3.62	4.80 6.88 1.52 2.22 2.84 4.30	4.46 4.42 0.90 1.34 3.52 4.18		31.6 47.2 70.0 156.0 5.0 2.6			24.9 25.8 46.0 50.0 7.5 5.2	21.6 23.7 50.5 59.9 4.9 6.2	24.0 11.2 46.1 50.5 3.6 6.9	20.9 15.9 67.5 42.7 5.8 7.4
8. GTT after adrenalin	4V 3V 2A 6A	0.72 1.34 4.18 4.18	0.96 1.86 5.06 4.30			0.72 0.98 3.72 2.83	0.58 1.00 3.32 2.23	0.86 0.68 1.11 1.18	0.80 1.38 0.58 0.97	11.9 5.4 11.7 12.1	7.9 3.9 9.0 11.8			10.3 7.8 11.1 9.0	12.4 6.1 10.1 5.2	8.9 5.5 2.2 4.1	7.8 5.2 1.9 1.9
9. GTT after altitude	3V 4V		0.46 0.90			0.70 0.78	1.04 0.68	0.64 0.58	1.36 0.40		34.8 32.2			31.0 14.1	17.5 17.6	16.9 21.4	7.4 27.2
10. Altitude alone (24,000 ft.)	1V 2V 3V 4V 1A 2A 3A 4A 5A 6A		1.80 1.18 0.58 0.92 0.52 1.16 0.78 0.58 1.08 0.54			2.40 1.78 0.84 1.76 0.64 1.18 1.00 0.80 0.94 1.54	2.74 2.46 1.44 1.70 0.70 ⁴ 0.74 ⁴ 1.80 ⁴ 1.02 ⁴ 1.14 ⁴ 1.60 ⁴	3.28 2.14 2.06 2.14 0.70 ⁴ 0.74 ⁴ 1.80 ⁴ 1.02 ⁴ 1.14 ⁴ 1.60 ⁴	2.86 2.14 2.06 1.88 1.88 1.88 1.88 1.88 1.88 1.88		53.5 39.3 27.2 34.1 16.2 6.4 9.5 20.0 15.0 10.0			58.7 56.0 38.8 30.2 12.7 4.7 11.6 15.3 21.9 12.6	59.9 40.4 34.4 49.3 12.7 4.7 11.6 15.3 21.9 12.6	40.1 46.1 42.0 35.0 12.1 ⁴ 10.6 ⁴ 9.4 ⁴ 17.1 ⁴ 18.6 ⁴ 15.0 ⁴	43.0 35.0 35.0 40.4 12.1 ⁴ 10.6 ⁴ 9.4 ⁴ 17.1 ⁴ 18.6 ⁴ 15.0 ⁴
11. Adrenalin alone	1V 2V 3V 4V	1.24 0.80	1.04 0.96 1.46			1.20 0.68 0.66 1.10	1.10 0.58 2.06 1.20	0.96 0.54 2.06 1.04	1.60 0.66 2.00 1.22	12.6 36.2 20.1 28.8	18.8 36.2 20.1 28.8			14.3 21.5 35.4 27.3	12.1 17.3 20.1 22.4	14.0 23.0 19.2 24.3	6.9 16.7 23.6 23.0

¹ V or A designates the sample being taken from the vein or the artery. U or E designates the adrenal cortical extract as being U—Upjohn's Adrenal Cortex Extract, or E—Eschatin (Parke, Davis and Company). ² Dog received only part of glucose to be injected. ³ Cortical extract and glucose were injected at the same time in this animal ⁴ 1½ hr. after B₂.

at ground level. Except in *dog 4*, with a very flat curve, the baseline values were never reached and two actually had begun to increase by the third hour. The lactic and pyruvic acid levels were elevated above those at ground level throughout the test, but no more than with exposure to altitude alone.

When glucose was given (*experiment 7*) with an infusion of adrenalin, the baseline was elevated a variable amount and throughout the test the blood glucose was maintained well above a normal fasting level. The dogs beginning the test with the highest baseline blood glucose values showed the least increment after administration, thus indicating that the renal threshold played some part. Blood lactic and pyruvic acids tended to be very high in both arterial and venous samples but when arterial samples were used, the lactic acid-pyruvic acid ratios did not change much throughout the experiment.

The response to glucose (*experiment 8*), after an infusion of adrenalin, showed the 30-minute increment to be less than during an ordinary glucose tolerance test, and the baseline was usually increased. Return to the baseline blood glucose value was rapid, essentially within one hour. Lactic acid values were extremely low at the end of the experiment as was the lactic acid-pyruvic acid ratio.

The response to glucose (*experiment 2*) at altitude, under the influence of adrenal cortical extract, showed that in the case of Eschatin², the injected glucose disappeared from the bloodstream extremely rapidly (within 15 minutes). With the Upjohn's extract, this was marked only in the case of *dog 3*, while *dog 2* offered the only example that was less marked. It should be noted that the blood lactic and pyruvic acid were very high at the one-hour level, which was not the case during the ordinary glucose tolerance test. In general, all the blood lactic acid and pyruvic acid levels were increased when glucose was injected or when the blood glucose began to show a secondary rise. *Dog 3* was interesting in that with both extracts it cleared its blood of glucose extremely rapidly with no secondary rise, but all with very high lactic and pyruvic acid levels. In all dogs, on being transferred from ground level to altitude, there was relatively little change in blood glucose.

The response to injected glucose at ground level under the influence of excess adrenal cortical extract (*experiment 3*) showed a low 30-minute increment indicating rapid clearing of the blood of glucose. The blood glucose was then either maintained or increased, so at 3 hours the level was above a normal one in all cases. In the 2 dogs on which lactic and pyruvic acids were determined, there was increase of these at the 3-hour levels.

The response to injected glucose (*experiment 9*), after exposure to 3 hours at 24,000 feet, was not markedly altered from that to glucose alone in two dogs studied.

Test Without Injected Glucose. Decompression to the equivalent of 24,000 feet (*experiment 10*) in only one case caused the blood glucose to go above a normal fasting level. All other cases, except one, exhibited blood glucose levels in the lower part of normal range immediately after the decompression. In all of these cases with relatively low blood glucose levels, the level gradually increased throughout the period of exposure while the two elevated ones decreased by 30-minutes to a lower

² Eschatin, an adrenal cortical extract, produced by Parke, Davis and Company.

and more normal level. No conclusions could be made about the lactic and pyruvic acid values except that they were extremely variable.

A constant infusion of adrenalin (*experiment 11*) maintained the blood glucose moderately above the baseline. Under adrenal cortical extract (*experiment 4*), decompression caused very little change in blood glucose level. Upjohn's Adrenal Cortex Extract (*experiment 5*) caused no rise in blood glucose level at the 15-minute period. In some cases, however, the 1-hour level was moderately elevated.

DISCUSSION

The level of blood glucose at a given time in an animal is the sum total of a great number of factors which are so great that when the blood glucose level is changed, extreme care must be exercised to ascertain just what factors have been altered. One of these, decrease in oxygen tension, has been shown to be potent in bringing about changes in the level of blood glucose (6, 7). However, there is little agreement as to its effect (6, 7); some report increase in the blood glucose levels (6, 7), some show no change, while still others show hypoglycemia. In addition to simply taking blood glucose levels, a great deal of additional information may at times be obtained by subjecting the animal to the stress of a suddenly added amount of glucose to find how it is utilized. This previously was done during exposure to simulated high altitudes and reported (1), but this in itself did not offer much in explanation of the phenomena observed.

In the reaction: $2 \text{ reduced DPN}^{\dagger} + \text{pyruvic acid} \rightleftharpoons \text{lactic acid} + 2 \text{ oxidized DPN}$, theoretically, the ratio of lactic acid to pyruvic acid will be determined by the ratio of reduced to oxidized DPN and therefore should reflect the level of oxygen in the tissues. This has been suggested by Friedemann and Haugen (9) who, under very carefully controlled conditions, did find small increases in humans in the lactic acid-pyruvic acid ratio at reduced atmospheric pressures.

In our experiments on dogs, we found that the same degree of significance could not be attached to the lactic acid and pyruvic acid values because of difficulty in keeping the animals quiet. This is brought out particularly when exposure to altitude alone (*experiment 10*) is studied. When venous samples were taken, there was extreme variation of both the lactic acid and pyruvic acid and lactate-pyruvate ratios throughout the test period. When arterial samples were taken, the results were much less variable but even then did not show levels significantly different from those at ground level.

At this point, it is interesting to note (*experiment 7*) the difference in results in taking venous or arterial samples when doing a glucose tolerance test and at the same time giving a constant infusion of adrenalin. Venous samples give extremely high and variable lactic acid and lactate-pyruvate ratios presumably because of the peripheral vasoconstriction and resultant stasis of the blood plus an increased glycolysis in the muscle tissue drained. The arterial samples, on the other hand, show fairly constant values for the lactate-pyruvic ratio, and the actual levels of the lactic and pyruvic acid values are what might be expected due to the added glucose and the

[†] Diphosphopyridinenucleotide.

glycolytic effect of adrenalin. Therefore, we have not taken credence in venous sample values but have accepted gross changes in values obtained from arterial samples.

Subjecting animals acutely to reduced oxygen tension has been shown to cause an increased production of adrenalin with a resulting increase in blood sugar (7). This effect of adrenalin occurs early; i.e., within 15 minutes after adrenalin is produced or injected. However, in our altitude experiments a gradual decompression was made to minimize the stimulation of the sympathico-adrenal system and on considering all studies at altitude, it is seldom seen that transferring the animals from the higher to the lower pressure caused an increase in the blood sugar. This is very much against the adrenal medulla playing any prominent rôle in the glucose tolerance at altitude. As can be seen from the fasting values, in all four cases where the standard glucose test was performed under decompression (*experiment 6*) the blood glucose was not elevated greatly by the decompression alone.

Consider now the remaining two effects together; namely, the effect of low oxygen tension on the various enzymes and the effect of adrenal cortical hormones. It has been shown in an associated study (10) that adrenal cortical extract enhances glycogen formation from glucose. In fact, it can be seen from *experiment 2*, table 1, that 0.5 gm. of glucose per kilogram of body weight is cleared from the bloodstream within 15 minutes. This does not rule out some smaller reduction of the action of enzymes due to lowered oxygen tension, but it is evident that at 24,000 feet, the enzymes responsible for glycogen formation from glucose are able to function rapidly and hormonal control plays much the pre-dominant rôle.

Furthermore, when the data are examined more carefully, one sees that the statement made in the previous paper (1) that the glucose tolerance of an animal during decompression is reduced is not the case. It will be noted that in no case was the 30-minute increment in blood glucose, after intravenously injected glucose, as high when the test was made at 24,000 feet as when it was made at ground level. This is also reported by Stickney, Northup and Van Liere (11). In other words, early in the test the glucose was actually taken from the blood stream more rapidly by the animal during hypoxia than when the corresponding test was done at ground level.

It has been shown that exposure to atmospheric decompression brings about stimulation of the adrenal glands with increased production of adrenal cortical hormones (12). The effect of these hormones in bringing about gluconeogenesis from protein and fat is well known (8). As can be noted from *experiment 5*, this gluconeogenic effect is evident in some cases one hour after injecting adrenal cortical extract while maximum effect has been shown to occur about the fourth hour after injection (13). Therefore, the plausible explanation of the altered glucose tolerance test performed during atmospheric decompression is that increased amounts of cortical hormones are produced by the adrenal cortex. This results in a more rapid uptake of glucose and formation of glycogen in the early stages of the test, but later gluconeogenesis from protein and fat cause the blood glucose to be raised and the increased level is prolonged. This effect can be duplicated by giving adrenal cortical extract before a glucose tolerance test at ground level and the effects of altitude are accentuated by giving the extract. It is conceivable that if the gluconeogenic effect

of adrenal cortical hormones (or extract) is somewhat delayed or not striking, that a very flat glucose tolerance curve could be obtained at altitude or after the injection of adrenal cortical extract. The data presented show that this occurred. One could go further and predict a flat glucose tolerance curve would be obtained from a well-fed animal that had been acclimatized and kept at altitude for some time. This was observed by Forbes (14) in two human subjects. This could be, because after an animal is kept at altitude for some time, the gluconeogenic effect of adrenal cortical hormones is balanced and the blood glucose returns to more normal levels (12). However, the glycogenic effect is apparently still active and Medvedeva (15) has actually isolated a factor from adrenal cortical extract which has a glycogenic effect with no effect on protein catabolism.

The blood glucose curve, after injected glucose at altitude or after injected adrenal cortical extract, varies from animal to animal depending upon individual variation in response to the glycogenic as opposed to the gluconeogenic effect of products of the adrenal cortex. For example; *dog 3*, in two tests done during atmospheric decompression after being given adrenal cortical extract, showed very little increase in his blood glucose level throughout the test even though markedly elevated lactic acid and pyruvic acid values indicated that gluconeogenesis was active. In this animal, apparently, the response to glycogenesis was more active in comparison to response to gluconeogenesis than was the case in the other animals observed. Should an animal be decompressed rapidly, excited, subjected to atmospheric decompression far beyond its tolerance or in any other way stimulated so that there is an increased production of adrenalin, then one would expect the glucose tolerance curve to be maintained at a higher level. This is probably true of *dog 1* in *experiment 6*, and also of one of the dogs in the previous paper (1).

SUMMARY AND CONCLUSIONS

The effect of adrenalin under various conditions was studied and indications are that stimulation of the sympathico-adrenal system plays only a minor rôle in most cases where a glucose tolerance test is performed on dogs at a simulated altitude of 24,000 feet. Apparently, there is sufficient oxygen at this altitude to allow functioning of the enzymes involved in glucose metabolism so that actually there is more rapid formation of glycogen than at ground level. The factor playing the predominant rôle is an increased production from the adrenal cortex. Although there is delayed return of the blood glucose to the baseline level after injecting glucose at a simulated 24,000 feet, careful perusal of the data presented indicates there is actually an *increase* in glucose tolerance. The injected glucose is converted to glycogen early in the test more rapidly than at ground level and the high glucose levels thereafter are due to gluconeogenesis from fat and protein brought about by the increased production of hormones by the adrenal cortex. Adrenalin may play a minor role in certain cases where the blood glucose level is maintained at markedly increased levels.

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ELECTROLYTIC RESISTANCE OF THE BLOOD CLOT

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THREE years ago investigations were undertaken to determine objectively the strength of clot retraction. For this purpose an insulated strain gauge was introduced into fluid blood anticipating that the force of clot retraction could be measured in terms of electrical resistance. A striking increase in ohms of resistance was observed, which, if due to the pressure of the retracting clot, would have indicated an impossibly large force of about 500 pounds per square inch.

Further consideration of this result led us to the conclusion that we were measuring the electrolytic resistance of the blood clot. As a consequence, studies of this phenomenon were undertaken. Papers to be published will detail the data accumulated in the past three years of study. The present paper deals with the technique for and the principles involved in the measurement of the electrolytic resistance of the blood clot.

METHOD

Ten ml. of blood is withdrawn slowly from the antecubital vein into a syringe which had been coated with Dri-film No. 9987.¹ A stop-watch is started when 5 ml. of blood are in the syringe. One ml. of blood is placed in each of two 13-mm. diameter glass tubes for the Lee-White clotting time at 37.5° C. Then 1.2 ml. of the blood is placed into each of three silicone coated glass cells for the measurement of the clot resistance (37.5° C.). Platinum electrodes are inserted into the middle of the blood sample.

The cells used to hold the blood are pyrex glass test tubes carefully selected for inside diameter within the limits of 0.447 and 0.450 inches. The test tubes are cut to an inside depth of approximately 1.050 inches. A cuff of plastic hugs the top of the cell securely (fig. 1). The cells are prepared with three coatings of Dri-film No. 9987 prior to each test.

The electrodes are constructed of platinum wire (0.020 inches thick) in the form of a circle with a horizontal cross-bar. The diameter of the circle is 0.211 or 0.172 inches (fig. 1). They are mounted on silver rods which, in turn, are mounted in a plastic cap through two holes fitted with set-screws. In this manner the electrodes can be accurately adjusted for depth and lateral position within the middle of the 1.2-ml. sample of blood. The electrode separation is 1.5 mm. The lead-in wires to the electrodes proper are first coated with one of the silicone resins (DC-804 or DC-996² and then with wax, if needed, for insulation.

The electrodes are connected into an A.C. bridge which incorporates a recording

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¹ Furnished through the courtesy of General Electrical Corp., Schenectady, N. Y.

² Furnished through the courtesy of the Dow-Corning Corp., Midland, Mich.

galvanometer for continuous automatic registration (fig. 2). For the initial determination of the point at which the resistance starts to leave the baseline resistance of the unclotted, fluid blood, the ratio arms of the bridge are set at 200 ohms each. This gives a full-scale deflection on the 10-inch strip chart of the recorder of 50 ohms for a maximum degree of sensitivity. For the recording of the entire clot resistance curve

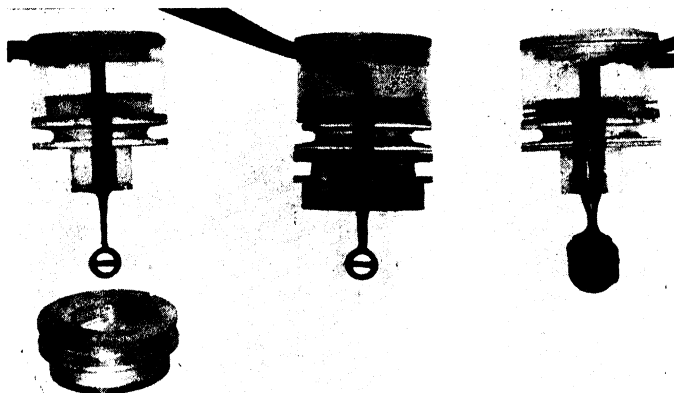


Fig. 1. ELECTRODES and blood cell; electrodes surrounded by the blood clot

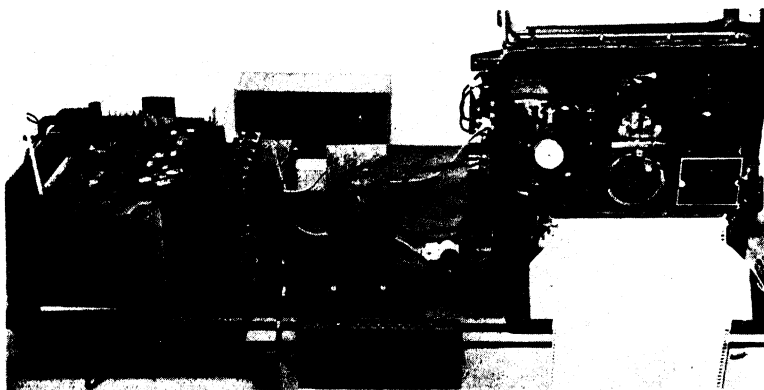


Fig. 2. *Left to right:* Water bath containing the blood cells and Lec-White tubes; telephone relay to which is connected a push-button panel for quick selection of any cell; bridge panel with bridge control knob, bridge selector switch and series resistance; recording galvanometer. In the background is a General Radio decade box which is automatically recorded on the strip chart.

over a 40-to 48-hour period, the ratio arms of the bridge are set at 5 ohms and 200 ohms to give a full-scale range of between 150 ohms and 1800 ohms. By means of a telephone relay 12 tests are recorded at one time. The instrument used in our work to date has been an old Leeds and Northrup 'pre-micromax' redesigned to use a 50-ohm slide wire and an A. C. galvanometer. A step resistance is inserted in series with the blood sample in order to place the measured resistance at a convenient place on the

strip chart, which serves the useful purpose of reducing the potential applied to the electrodes.

The bridge supply voltage used is 0.5 volts at 60 cycles. Resistance measurements at 60 cycles have been found to vary little from those at 1000 cycles. However, it is essential to platinize the electrodes quite heavily in order to avoid polarization effects which may make the initial determinations slightly unstable. No effects of the applied potential as high as several volts have been detectable.

Before the start of each experiment, the electrodes are plated with platinum black for 3 minutes and then tested in 0.01N KCl to insure resistance values that are within 3 per cent or less of each other.

The curve of resistance of the blood clot over a 40- to 48-hour period has been termed 'the electrolytic resistance curve'.

CRITIQUE OF METHOD

The term 'electrolytic' is used rather than electrical because the measurements are made in a conducting solution, the blood serum. The term 'resistance' is used, however, in preference to that of conductivity because what is measured is the resistance offered by the clotting and clotted blood to the passage of current through the serum.

For the purposes of the studies herein reported, the electrodes were first made 0.211 inches in diameter. Two types of electrodes were studied, the first of sheet platinum but circular, the second of wire of the design illustrated in figure 1. It was found that two factors were most important for the accurate determination of the clot resistance. These are: 1) the relationship of the size of the electrodes to the volume of the blood clot and 2) the nature of the walls of the cell in which the blood is contained. Consequently, an analysis of these two factors is included in this paper.

Relationship of the Size of the Electrodes to the Volume of the Blood Clot

This ratio is important because of the nature of the current flow between the electrodes and because the size of the blood clot varies in different clinical conditions.

a) *Current flow between the electrodes.* Since the blood clot is a poor conductor and, after clot retraction, is surrounded by serum which is highly conducting, it is essential to consider the inevitable escape of current out of the clot into the serum. This leakage of current has been observed to be a variable factor from blood to blood.

The pathways over which current flows between the electrodes consist of two parallel circuits, an internal pathway between the inner faces of the electrodes (resistance r), and an external pathway between the outer faces of the electrodes (resistance r'). These pathways of current flow are diagrammed for plate electrodes in figure 3.

In order to determine the resistance values of these pathways, experiments were performed with three types of circular plate electrodes (of diameter 0.211 inches): 1) with both faces of the electrodes electrically active, thus measuring the total electrolytic resistance (R); 2) with their outer surfaces waxed, thus measuring only

the internal resistance (r); and 3) with inner faces waxed and blocked, thus measuring only the external resistance (r'). Comparative readings show (table 1) that the external resistance (r') is considerably greater than the total resistance (R) or the internal resistance (r). Measurements of the resistance in saline of varying concentrations show that the behavior of the electrodes in both saline and clotted blood is essentially the same, but with one distinguishing difference. The external resistance, r' , in the blood clot system is much greater in proportion to the R and r values than in the saline, indicating that the blood clot plays a significant rôle in the resistance observed. The r value for the blood clot is 10 times the R value, whereas the r' in saline is only three times as great as R .

The fact that a leakage of current takes place through the serum surrounding the blood clot is evidenced by the increase in resistance observed when the serum surrounding the clot is removed (table 2). This can be accomplished simply by

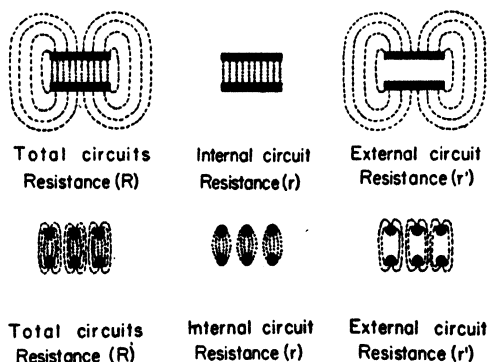


Fig. 3. *Upper*: CURRENT FLOW between plate electrodes; *lower*: current flow between wire grid electrodes.

lifting the electrodes with their clot out of the serum. As seen in table 2, the increase in resistance in the R value, the 'loss factor,' is of the order of 8 per cent to 11 per cent and for r is of the order of 5 per cent to 6 per cent, whereas the increase in resistance of the external pathway, r' , varies from 25 per cent to 57 per cent. It is evident, therefore, that a major loss in resistance occurs because of leakage of current through the serum surrounding the blood clot.

In an attempt to reduce this factor, comparable studies were made utilizing grid electrodes. The grid structure allows the current to flow around each wire (fig. 3) which provides a much shorter external pathway. In this case the 'loss factor' for r' is of the order of 9 per cent to 15 per cent as compared with the resistance loss factor of 26 per cent to 57 per cent for the plate electrodes (table 2). The changes in total measured resistance (R) are of the order of 7 per cent to 9 per cent as compared with 8 per cent to 11 per cent for the plate electrodes where the total measured resistance (R) was 200 ohms or less. No tests were made with conventional square electrodes, as used in other studies, as it must be immediately apparent that the corners of square electrodes of the same surface area would project closer to the surface of the blood clot, and result in larger losses of resistance. Round plate electrodes,

coated on the backs, were not considered because it was found that they furnished a poor support for the blood clot and introduced an inactive surface which could precipitate clotting.

In hundreds of routine determinations taken at various points along the course of the electrolytic resistance curve, the loss factor for R with grid electrodes was of the order of 6 per cent to 9 per cent when the resistance was approximately 400 ohms or less, and 11 per cent to 13 per cent when the resistance was between 400 and 800 ohms. (The clot volume was normal in both cases.)

b) *Ratio of 'electrode size' to blood sample volume and clot volume.* The data in (a) hold only when resistance measurements are below 800 ohms and the volume of the blood clot is normal. Clearly, reduction of the clot volume surrounding the electrodes would bring the electrodes closer to the serum and increase the loss factor; whereas increasing the clot volume would have the opposite effect. As noted initially, the

TABLE 1. COMPARATIVE VALUES OF TOTAL RESISTANCE (R), INTERNAL RESISTANCE (r) AND EXTERNAL RESISTANCE (r') FOR PLATE ELECTRODES OF DIAMETER 0.211 INCHES

TIME	IN BLOOD CLOT			SALINE CONC.	IN SALINE		
	R	r	r'		R	r	r'
<i>hr.</i>				%			
$\frac{1}{2}$	150	165	485	0.9	19	21	62
1	139	140	988	0.09	173	196	556
2	144	145	1768	0.045	339	383	1028
$2\frac{1}{2}$	148	147	1365	0.0275	672	759	2088
4	150	148	1393				
20	193	165	1445				
30	172	157	845				

blood sample volume is 1.2 ml. contained in a tube of inside diameter 0.447" to 0.450". The diameter of each electrode is 0.211". With a separation of 1.5 mm., the maximum or diagonally measured distance between the two is 0.556 cm. The volume of a theoretical sphere of this diameter is 0.0899 cu. cm. The volume of the blood sample 'sphere' having the mean diameter (0.449 inches) is 0.776 cu. cm.³ The ratio of the electrode 'sphere' to the blood sample 'sphere' (0.0899 cu. cm. to 0.776 cu. cm.) is 1 to 8.6. The percentage volume of clot on the electrodes (with as many red cells as it will retain) in normal blood and in a variety of diseases, was found to be 46 per cent of the blood sample. Taking 50 per cent as an approximate figure, the ratio of the electrode 'sphere' to the clot 'sphere' is then approximately 1 to 4.3. For these ratios the error due to the 'loss factor' is, as indicated, of the order of 6 per cent to 13 per cent when the total measured resistance is under 800 ohms, using grid electrodes.

In untreated polycythemia vera it has been found that, irrespective of the red cell count, the percentage clot volume shrinks to remarkably small values of from

³ The reason for taking the spherical volumes rather than the actual volumes of the blood and clot is that the current will take the shortest radial path through the clot.

12 per cent to 25 per cent of the initial blood sample volume. Here the ratio of electrode to clot volume may drop to 1 to 2 or lower, and the loss factor (in a large number of experiments with grid electrodes of diameter = 0.211") mount to between 22 per cent and 47 per cent. This is accompanied by a marked increase in clot resistance to between 800 and 1000 ohms (as will be described fully in a subsequent paper). This increased error is compounded of at least two factors: first, the loss due to a reduction in the size of the clot; and second, a loss which is accounted for by the increase in clot resistance. The formula for parallel circuits, $R = r \cdot r' / r + r'$ indicates that as r increases, the same change in r' will exert a greater effect upon the value for R .

TABLE 2. LOSS FACTOR WITH ELECTRODES OF 0.211 INCHES DIAMETER; INCREASE IN RESISTANCE ON REMOVAL OF THE SURROUNDING SERUM

EXPER.	TIME	R	INCREASE		r	INCREASE		r'	INCREASE	
			Ohms	%		Ohms	%		Ohms	%
	hr.									
Plate Electrodes of 0.211 Inches Diameter										
80	12	265	25	9						
	23	287	28	10				1855	715	39
	46	198	16	8				637	163	26
81	4	155	10	6	148	6	5			
	20	193	21	11	165	8	5	1445	830	57
	30				157	10	6			
Grid Electrodes of 0.211 Inches Diameter										
	2 $\frac{1}{2}$	167	13	8				430	40	9
		190	13	7				600	85	14
	19	184	17	9						
		218	15	7				780	115	15

This increase in loss of resistance due to leakage of current through the serum at high clot resistance levels becomes, therefore, an important consideration whenever the measured clot resistance increases above 500 to 800 ohms. This loss may be reduced by either increasing the volume of the blood sample (and consequently the volume of the clot) or by reducing the size of the electrodes. Since it is desirable to keep the blood sample small, plate and grid electrodes of a diameter of 0.172" \pm 0.004" were tested in the same blood sample of volume 1.2 ml. with a separation of 1.5 mm. The ratio of the electrode sphere volume to blood sample volume was thereby increased from 1 to 8.6 to that of 1 to 15 (ratio of electrode to normal clot volume increased from 1 to 4.3 to 1 to 7.5). Tests were also run with this size grid electrode at a separation of 1.0 mm. with a ratio of 1 to 16⁴. Table 3 gives the comparative values of the loss factors and the electrode to clot volume ratios for the two different sizes of electrodes. Irrespective of the electrodes used, a drop in ratio to below 1 to 3 is

⁴ The results are similar to those with the same grid at a separation of 1.5 mm. and are, therefore, included in that column for simplicity.

accompanied by a sharp increase in resistance loss. Also, the resistance loss is greater with the plate electrodes than with the grid electrodes although the difference is significant only in the higher resistance range.

The efficiency of the electrodes appears to be measurable in yet another manner, namely, by comparing the specific resistances obtained with each type in the same (aliquot) blood sample. While the measured resistances will vary, the specific re-

TABLE 3. COMPARISON OF 'LOSS FACTOR' AND THE ELECTRODE TO CLOT VOLUME RATIOS

SUBJ.	DIAM. = 0.211"				ELEC./ CLOT RATIO	DIAM. = 0.172"				ELEC./ CLOT RATIO
	Plate elec.		Grid elec.			Plate elec.		Grid elec.		
	Ohms	% loss	Ohms	% loss		Ohms	% loss	Ohms	% loss	
M.F.			117	6.6	1/5.0			269	4.0	1/9.3
H.H.			393	14.0	1/4.8	528		978	1.5	1/8.4
F.T.			109	7.4	1/4.8	172	5.9		5.9	1/8.4
J.B.			173	11.5	1/4.6			298	4.5	1/8.6
M.G.			225	9.2	1/4.6	374	4.5	473	3.3	1/7.9
E.M.			250	7.6	1/4.6			445	5.6	1/8.6
W.D.			431	6.0	1/4.6	472	4.4			1/8.1
C.T.			229	8.4	1/4.5			265	2.9	1/8.3
E.M.			347	7.0	1/4.4			327	5.3	1/8.2
W.D.	390	8.6	385	6.7	1/4.3					
C.W.	298	12.6	410	13.6	1/4.3					
R.H.	236	6.8	251	8.5	1/4.1					
W.S.	282	8.9	265	8.6	1/4.1	374	5.9			1/7.2
A.M.	65	3.3	118	4.2	1/4.0					
D.K.			165	5.8	1/4.0	271	6.3			1/6.9
C.R.			517	9.8	1/4.0			918	10.3	1/6.9
M.N.			306	11.7	1/3.9			585	17.5	1/7.2
K.F.			528	5.2	1/3.5	1356	8.6	1942	11.3	1/6.2
K.F.	771	24.8	781	16.5	1/2.8	1304	11.9			1/5.0
K.F.	761	32.1	955	15.7	1/2.8	1248	10.1			1/4.8
A.V.			219	6.4	1/2.4			220	3.3	1/4.5
A.T.	173	13.1	241	23.8	1/2.0	461	7.3			1/3.5
C.R.			673	32.1	1/1.5			800	33.1	1/1.5
C.R.	489	24.4			1/1.1			733	23.5	1/1.9

sistances should be comparable. It will be noted in table 4 that generally the specific resistances for the smaller electrodes (both grid and plate) are greater than those for the larger electrodes. The specific resistances in the fluid, unclotted whole blood are comparable for all electrodes.

When the specific resistances obtained with the small grid electrodes are plotted against those for the large grid electrodes, the curve yields values that for the small grid are 50 per cent greater than those for the large grid. In like manner the curve for the small plate resistances against those of the large grid electrodes yields values for the plate electrodes that are 20 per cent greater. It seems reasonable to conclude, therefore, that the ratio of the size of the electrodes to that of the blood sample is of

primary importance and should not be much smaller than 1 to 8.6 and, possibly, should be of the order of 1 to 15. Also, the wire grid electrodes appear preferable to the plate electrodes of the same size partly because they yield a firmer anchor for the blood clot.

Influence of the Nature of the Cell Walls and Clot Retraction upon Clot Resistance

It has been found true, also, that disturbance of the clot during its formation and interference with clot retraction may cause significant reductions in the clot resist-

TABLE 4. SPECIFIC RESISTANCE AS DETERMINED WITH VARIOUS ELECTRODES

SUBJ.	ELEC. D = 0.211"		ELEC. D = 0.172"	
	Plate elec.	Grid elec.	Plate elec.	Grid elec.
M.F.		341		596
H.H.		1146	1203	2168
F.T.		318	394	
J.B.		504		661
M.G.		656	852	1049
E.M.		729		986
W.D.		1256	1075	
C.T.		668		588
E.M.		1012		725
W.D.	1292	1123		
C.W.	988	1195		
R.H.	782	732		
W.S.	934	773	852	
A.M.	215	344		
D.K.		481	618	
C.R.		1506		2035
M.N.		892		1297
K.F.		1540	3089	4310
K.F.	2552	2277	2970	
K.F.	2520	2782	2842	
A.V.		638		488
A.T.	573	702	1050	
C.R.		1962		1774
C.R.	1620			1625

ance. Stirring of the blood prior to the introduction of the electrodes causes a marked loss in clot resistance. Should the electrodes be removed from the blood after a portion of the clot has formed and then be reintroduced, the clot resistance will level off at that point and rise no farther. The initial clot which clings to the electrodes is surrounded by a second clot that can be peeled off like the layers of an onion. However, after a clot has formed covering the electrodes, the blood may be inverted gently without disturbing the subsequent clot formation or the clot resistance rise. In addition, the redistribution of red cells so induced does not seem to change the clot resistance even where the sedimentation rate is sufficient to settle the red cells prior to complete clot formation.

When the cell wall, irrespective of the material of which it is made, is roughened

so as to induce adhesion of the clot to it, a marked reduction in clot resistance frequently occurs. Conversely, coating of the pyrex blood cell with Dri-film no. 9987, which is highly water repellant, results in a 15 per cent greater clot resistance than in the untreated glass cell (18 experiments, 54 tests). The results with the untreated cells were often erratic and in some cases the clot adhered to the cell walls. In comparative studies when the cells were made of roughened lucite, lucite coated with DC-200 fluid or Dri-film no. 9987, and glass coated with Dri-film no. 9987, the results in the glass Dri-film coated cells were almost always higher and more consistent (table 5). In addition to the above, some tests were also made of DC-802 resin, DC-995 varnish and DC-1107 on pyrex glass. These also yielded lower results and had the added disadvantage, even though they gave a permanent coat, of being easily scratched. In routine tests the Dri-film is wiped on, washed thoroughly, and then wiped dry and polished. This procedure is repeated three times prior to each test.

In order to determine the accuracy of the method, multiple tests were run on the same blood sample and on the same individual at different times. Measurements

TABLE 5. EFFECT OF VARIOUS CELL SURFACES ON ELECTROLYTIC RESISTANCE OF THE CLOT

TEST	LUCITE ROUGH	LUCITE DC-200	LUCITE DRI-FILM	GLASS DRI-FILM
1		136	192	
2	125		191	221
3	222			338
4			330	348
5			376	448

were taken at the maximum clot resistance from which the initial resistance of the fluid unclotted blood was subtracted. All tests were performed in triplicate. The deviations of the three individual measurements from the mean of the three was then taken. From the deviations so obtained in a large number of tests, the standard deviation (S.D.) was calculated.

In 128 tests in which the mean clot resistances were between 150 and 350 ohms, the S.D. so calculated was ± 18.5 ohms. In the higher resistance range, between 500 and 900 ohms, the S.D. was ± 54 ohms (46 tests). The error is greater in the higher range for reasons given. This increase in error with an increase of the clot resistance is, however, always negative, as is the loss factor upon which it presumably depends, so that the validity of any interpretations based upon an increase in resistance above normal is not impaired.

A comparison of repeated tests performed upon the same individual (normal and otherwise) performed at intervals, but while the physiological status of the subject appeared to be stable yielded data of the same order of magnitude as the agreement between triplicate determinations on the single sample of blood (table 6).

DISCUSSION

The technique for the determination of the electrolytic resistance of the blood clot must take into consideration certain circumstances under which the resistance is measured. These circumstances are: a) a relatively low conducting blood clot whose

resistance is to be measured, and *b*) the presence of a highly conductive layer of serum surrounding the clot after clot retraction has occurred. The conductivity of the serum may be as much as 50 times that of the blood clot, so that leakage of current through the serum must be held to an irreducible minimum.

This 'loss factor' is dependent upon two factors: 1) the manner in which current flows between the electrodes and 2) the ratio of the size of the electrodes to the volume of the blood clot. To understand the former it is convenient to picture the current flow as consisting of two parallel circuits, an internal pathway between the inner faces of the electrodes and an external pathway between the outer faces. Analysis of these pathways separately reveals that the 'loss factor' is due, at least in part, to a leakage of current over the external pathway, i.e., a leakage of current into the serum from the backs of the electrodes. The loss, with plate electrodes, over the external pathway is seen to be of the order of 26 per cent to 57 per cent. When the electrodes

TABLE 6. COMPARISON OF REPEATED TESTS ON THE SAME INDIVIDUALS

NORMALS		ABNORMALS	
Subj.	Ohms	Subj.	Ohms
<i>E.B.</i>	334, 327	<i>C.Rl.</i>	882, 797
<i>J.T.</i>	344, 439	<i>I.L.</i>	852, 899
<i>I.H.</i>	165, 161, 133	<i>H.W.</i>	278, 235, 296
<i>E.F.</i>	124, 155, 149	<i>S.M.</i>	908, 845
	137, 132, 153	<i>C.R.</i>	770, 702
<i>V.S.</i>	174, 216, 192, 200	<i>R.G.</i>	631, 655
	222, 247, 268, 172	<i>D.L.</i>	192, 211
<i>C.W.</i>	426, 410	<i>C.J.</i>	350, 338, 327, 358
		<i>D.R.</i>	179, 196
		<i>A.H.</i>	16, 25, 14
		<i>W.D.</i>	392, 366, 385

are in the form of a wire grid, so that the external pathway is shortened considerably, the loss in this circuit drops to approximately 9 per cent to 15 per cent. In addition, the grid is an efficient anchor for the clot. Under otherwise identical conditions, the wire grid structure seems to be preferable to a solid plate.

Of primary importance in minimizing the leakage of current into the serum is the maintenance of an adequate volume of clot surrounding the electrodes. In order to compare the size of the electrodes to that of the clot and also the blood sample, the spherical volume of each was calculated. With these indices, four types of conditions were studied: 1) plate and grid electrodes with a diameter of 0.211" and a ratio of electrode volume to blood volume sample volume of 1 to 8.6 and 2) plate and grid electrodes of diameter of 0.172", with a ratio of 1 to 15. Under normal conditions, the ratios of the electrode volume to that of the clot volume were 1 to 4.3 and 1 to 7.5, respectively.

It was evident that for resistance values below 400 to 500 ohms, there were only small differences in the loss factor and these were in favor of the smaller grid electrodes. But above these limits a marked disparity appeared. The larger electrodes of

ratio 1 to 8.6 showed appreciably greater losses over those of the small electrodes of ratio 1 to 15. The losses, also, were greater with the plate electrodes than with the grid electrodes.

Also of great importance is the fact that the loss of resistance is not a constant factor, but increases as the measured resistance increases. This phenomenon is dependent upon the law of parallel circuits, as expressed in the formula, $R = r \cdot r'/r + r'$, and on the fact that the amount of clot 'shielding' the electrodes from the serum decreases in certain instances as the clot resistance increases. In polycythemia vera the percentage clot volume becomes extraordinarily small, 12 per cent to 25 per cent as compared with the normal percentage clot volume of 46 per cent. Under these circumstances the ratio of the electrode to clot volume drops below 1 to 3, when the electrode to blood sample volume is 1 to 8.6, and large losses in resistance are encountered. When the electrodes are of ratio 1 to 15, the losses are reduced proportionately. Fortunately, these reductions in clot volume have been found almost exclusively in polycythemia vera where extremely high clot resistances are also encountered. Consequently, since the loss factor is larger only when the resistance measurements are greater the essential validity of the data is not impaired.

In addition, comparison of the specific resistances obtained with the above electrodes showed that the specific resistances are greater with the grid electrodes and when the amount of clot is large, i.e. at the ratio of 1 to 15. These variations in specific resistances also indicate that the 'loss factor' is reduced with grid electrodes and when the larger amount of clot surrounds the electrodes. Hence, it is clear that the ratio of the electrode volume to that of the blood sample should be at least as large as 1 to 8.6, and that grid electrodes are probably preferable to plate electrodes.

The above data also indicate that the specific resistance cannot be accurately calculated by the conventional formula applicable to solutions of homogeneous makeup, especially in polycythemia vera.

In recent studies (1) of the 'electrical resistance' of the blood clot, the authors use square electrodes of $\frac{1}{4}$ sq. cm. in area, spaced 5.0 mm. apart in a blood volume of 1.5 ml. having a diameter of 12 mm. Applying the calculations used in our study, the ratio of their electrode volume to that of the blood sample volume is between 1 to 5 and 1 to 2.27 and the ratio of electrode to blood clot volume is between 1 to 2.5 and 1 to 0.64 or less. At these ratios large losses of resistance are unavoidable. In addition, the authors present their figures in terms of specific resistance. Moreover, they determine the resistance for periods of less than an hour, so that the clot resistance (at the maximum of the electrolytic resistance curve) was not determined. The specific resistance of the normal fluid blood in both their studies and ours are of the same order.

The accurate determination of the electrolytic resistance of the blood clot is also dependent upon the orderly and uninhibited retraction of the clotting blood. Experiments in which clot retraction is interfered with all show a reduction in the measured clot resistance. Roughening of the walls of the cell, thereby increasing adhesion of the blood to the cell walls, causes a variable and significant lowering of the clot resistance. This factor is enhanced when plate electrodes are used in place of grid electrodes.

By the use of silicone compounds as coating for the cell walls the adhesion of the blood to the cell walls can be materially reduced. Various silicones tested have shown different effects. Of the compounds tried, Dri-film no. 9987 best prevented the adhesion of the blood to the cell walls and yielded the most consistent measurements and the highest clot resistances. Hence, the coating of the cells with Dri-film no. 9987 has been adopted as standard procedure in our studies.

The results of measurements of the clot resistance on multiple samples of a given blood and of multiple measurements on the blood of the same individual at different times indicate that reasonably accurate and reproducible results are possible.

SUMMARY

A technique for the determination of the electrolytic resistance of the blood clot and an analysis of the important aspects is presented. The key features of this technique are as follows: *a*) The use of a recording galvanometer to secure the complete electrolytic resistance curve over a 40- to 48-hour period. *b*) Electrodes constructed of wire in a circular grid pattern are preferable to solid plate electrodes of more conventional design. *c*) The ratio of electrode size to the blood sample volume (as determined by the ratio of the 'spheres' with their respective diameters) must not be less than 1 to 8.6 and preferably closer to 1 to 15. *d*) It is essential that there be a minimum of interference with the orderly retraction of the blood clot. Coating of the cell walls with Dri-film no. 9987 seems to come closest to fulfilling this requirement.

Thanks are due Mr. George Gebhart, engineer, and Mr. Hubert E. Blackburn whose cooperation during the early phases of this work was invaluable.

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ELECTROLYTIC RESISTANCE OF THE BLOOD CLOT: RESISTANCE CLOTTING TIME, ONSET OF CLOT RETRACTION AND THE CLOT RESISTANCE

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IN THE previous paper a technique for the determination of the electrolytic resistance of the blood clot was presented (1). The present paper deals with the variations in the electrolytic resistance of the blood clot over a 40- to 48-hour period, and with the physiological significance of certain measurements of the resistance. The curve of the resistance against time will be called the electrolytic resistance curve (E.R.C.).

PROCEDURE AND RESULTS

Electrolytic Resistance Curve (Fig. 1). PHASE 1. The initial rise in resistance above the baseline resistance of the fluid blood is associated with the initial precipitation of fibrin about the electrodes. The elapsed time between the taking of the blood and this point is called the resistance clotting time.

PHASE 2. There follows a rapid rise in resistance over one to two hours. This is the primary rise.

PHASE 3. Under certain conditions, a sharp break in the primary rise may be induced. This point indicates the onset of clot retraction. It is called the primary peak.

PHASE 4. Subsequent to the primary rise there is a secondary slower rise in resistance which may reach its maximum at any time within 20 hours. The maximum resistance measured minus the fluid blood resistance is the clot resistance.

PHASE 5. During the final 20- to 24-hour period a rapid fall in resistance usually occurs.

The aspects of the curve which appear to have the greatest significance are the resistance clotting time, the clot resistance and the primary peak.

As noted (1), since the specific resistances cannot be calculated, the experimental conditions under which the resistance measurements have been made must be stated. These are: 1) electrodes constructed of wire 0.020 inches thick, circular in shape of diameter 0.211 inches, with a horizontal cross-bar; 2) electrode separation of 1.5 millimeters; 3) blood sample cell of pyrex glass with an inside diameter between 0.447 and 0.450 inches, coated with Dri-film no. 9987.

Resistance Clotting Time (R.C.T.). The initial onset of the rise in resistance above the base-line resistance of the unclotted whole blood has been termed the resistance clotting time (R.C.T.). At this time the electrodes are coated with fine strands of fibrin whereas the surrounding blood is still fluid. As the resistance rises a clot grows

upon the electrodes. This growth is indicated by the following observations obtained by removing one of several electrodes from the clotting blood at successive intervals:

- 8'33" R. C. T. A few fine strands of fibrin on the electrodes.
- 10'10" Electrodes plated with a fine layer of fibrin.
- 12'00" A fine clot has formed between the electrodes.
- 14'00" Clot has thickened somewhat around the electrodes.
- 16'00" Clot several millimeters thick around the electrodes.
- 22'00" Clot about twice as large as in previous observation, has assumed globular shape.
- 25'00" Clot larger, less than a third of final clot size. At all times the remainder of the blood is fluid and without fibrin strands.

Of key significance is the fact that at the R.C.T. a minute amount of fibrin is present only at the electrodes and not elsewhere in the blood. Hence, this point in time, the R.C.T., must measure the initial onset of clot formation in whole blood.

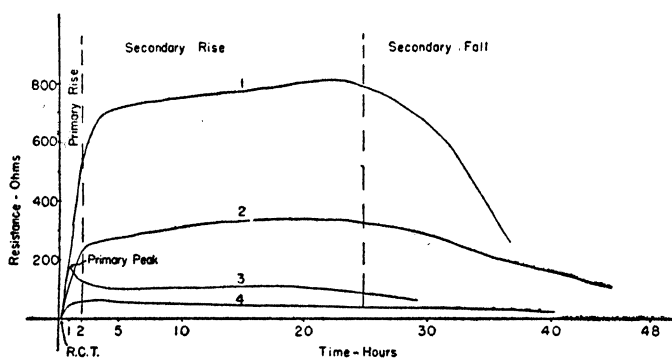


Fig. 1. ELECTROLYTIC RESISTANCE CURVE. Curve 1: abnormally high curve. Curve 2: normal curve for male. The normal curve for the female is the same shape but lower. Curve 3: illustrating the primary peak. Curve 4: abnormally low curve.

Tests performed with various sizes of electrodes and currents indicate that within limits the R.C.T. is independent of the size of the electrodes and of the current flow between the electrodes.

Normal R.C.T. In 21 normal white adult males the mean R.C.T. was 10.3 ± 1.0 minutes; in 21 adult white females the R.C.T. was $9.5 \pm .94$ minutes. The probable error of the difference between the two means is 12.12 seconds. Since the difference between the means is close to four times the P.E. it is difficult to say that a significant difference between the R.C.T.'s exists although it may. The mean R.C.T. for the 42 adult humans is 9.9 minutes (table 1).

In 3 menstruating females tests were run at intervals over a complete menstrual cycle. No variations in R.C.T. were discernible.

Comparisons of the R.C.T. with the Lee-White clotting time, measured in 13-mm. diameter tubes at 37.5°C ., show a significant correlation between the two types of tests. The coefficient of correlation is 0.696 with a P.E. of 0.0580.

Resistance of the Fluid Unclothed Whole Blood. Before proceeding with the consideration of the clot resistance the nature of the fluid blood resistance should be evaluated. In 21 normal males the resistance of the fluid blood, just prior to the in-

ception of clotting, was 80.8 ± 7.0 ohms. In 21 normal females the fluid blood resistance was 67.1 ± 4.8 (table 1). The P.E. of the difference between the two means is 1.249. The difference between the means is considerably greater than four times the P.E. so that the two means are significantly different. Since the fluid blood is a homogeneous medium the specific resistances may be calculated.¹

Clot Resistance. From the maximum measured resistance the resistance of the fluid blood is subtracted to obtain the clot resistance (fig. 1). Considerable thought was given the question whether to record the total measured resistance or the clot resistance. In some instances no increase in resistance above that of the fluid blood is observed. In thrombocytopenic purpura, the clot resistance is extremely small. Following splenectomy the clot resistance increases but the fluid blood resistance remains unchanged. The fluid blood resistance and the clot resistance do not appear to have any direct relationship. It would appear, therefore, that the processes responsible for the clot resistance are superimposed upon those of the fluid blood resistance and are, to a greater or lesser degree, independent of them. Hence, it would seem that the clot resistance more accurately mirrors the behavior of the clot than does the total resistance.

In the normal human a marked difference in the clot resistance is observed between adult white males and females. In 21 presumably normal males between the ages of 23 and 56 years the mean clot resistance was 311 ± 44.4 ohms.² In 21 normal white females between the ages of 22 and 50 years the mean clot resistance was 179 ± 33.5 ohms. All these had normal menstrual periods with the exception of 2 (ages 44 and 50) who had passed their menopause and one (age 39) who had had a hysterectomy 8 years previously. In 2 female subjects the clot resistance was determined at weekly intervals over a 6- to 8-week period and in a third at 2-week intervals. There was no detectable variation in the clot resistance with the menstrual cycle as there was none in the R.C.T. The percentage clot volume in the male was 46.1 per cent and in the female, 45.7 per cent, averaging 45.9 per cent (table 1). This clot volume is of interest since the clot resistance is so different.

Considerable differences in resistance have also been found to exist in various clinical disease states. In table 2 are listed a range of unselected clot resistances and other hematological data. At first glance there would appear to be no significant correlation between either the red cell count, the platelet count, the white cell count or the percentage clot volume and the clot resistance. The highest resistances are associated with the highest red cell counts because, as will be noted later, these occur in polycythemia vera.

Of major interest is the possible existence of a relationship between the clot resistance and the concentration of platelets. From table 2 there would appear to be little relationship between these two phenomena. Of particular note are the low resistances of 115, 131, 195 and 197 ohms in association with platelet counts over 1,200,000.

However, in view of the fact that the behavior of the clotting mechanism, and

¹ Specific resistance = Measured resistance/K. $K = 0.001754 \times \text{resistance of electrodes in } 0.01N \text{ KCl at } 37^\circ \text{ C.} = 0.344.$

² All \pm figures indicate standard deviations.

hence the clot resistance, may depend on the platelet concentration, it becomes important to analyze the data in different ranges of clot resistance and platelet concentrations. Within an arbitrary range of clot resistance below 300 ohms and a platelet count below 400,000 (100 cases) a significant statistical correlation appears to exist between the two phenomena. In this range the coefficient of correlation is 0.58 and the standard error is 0.10. Similar measurements are needed in the ranges above those studies, although, at present, sufficient data are not yet available.

TABLE 1. NORMAL VALUES

MALE						FEMALE					
Subj.	Age	R.C.T.	Resistance		Clot vol.	Subj.	Age	R.C.T.	Resistance		Clot vol.
			Fluid blood	Clot					Fluid blood	Clot	
	yr.		ohms	ohms	%		yr.		ohms	ohms	%
<i>E.B.</i>	46	10'25"	91	331	55.8	<i>E.F.</i>	26	9'38"	61	142	39.2
<i>S.G.</i>	25	12'10"	89	281		<i>V.S.</i>	24	9'24"	69	211	45.8
<i>M.P.</i>	32	11'04"	84	318		<i>E.N.</i>	29	8'33"	58	148	33.3
<i>M.Pz.</i>	40	9'38"	77	351		<i>R.C.</i>	28	9'08"	60	139	
<i>L.C.</i>	56	9'15"	87	276	50.0	<i>I.H.</i>	32	10'13"	62	153	44.2
<i>S.S.</i>	38	9'55"	82	286	45.8	<i>D.C.</i>	22	10'30"	66	154	60.8
<i>R.L.</i>	32	10'12"	86	299	52.5	<i>C.M.</i>	44	8'35"	73	229	58.4
<i>G.K.</i>	47	8'37"	81	305	50.0	<i>H.L.</i>	26	9'42"	63	153	41.7
<i>R.M.</i>	24	9'30"	84	407	39.2	<i>A.K.</i>	50	11'13"	72	198	39.2
<i>B.W.</i>	23	10'12"	82	354	47.5	<i>B.M.</i>	40	11'42"	62	130	43.3
<i>E.A.</i>	28	9'55"	81	309	41.7	<i>G.E.</i>	25	10'53"	71	170	50.0
<i>I.H.</i>	22	10'10"	66	340	43.3	<i>N.G.</i>	24	10'05"	66	230	41.7
<i>L.S.</i>	49	11'17"	90	357	58.4	<i>E.G.</i>	31	10'23"	72	166	52.5
<i>D.E.</i>	26	11'15"	78	390	41.7	<i>L.M.</i>	25	8'25"	70	253	43.4
<i>R.C.</i>	27	10'25"	74	277	39.2	<i>G.M.</i>	45	9'05"	68	183	51.7
<i>J.M.</i>	24	10'48"	78	307	41.7	<i>M.M.</i>	34	10'07"	62	173	50.0
<i>D.M.</i>	27	8'58"	70	278	33.3	<i>T.B.</i>	30	10'12"	62	217	47.5
<i>R.H.</i>	25	9'07"	75	251	48.3	<i>E.R.</i>	39	11'28"	70	197	44.2
<i>W.S.</i>	25	10'00"	74	265	47.5	<i>E.S.</i>	28	9'30"	66	190	44.2
<i>M.D.</i>	36	12'38"	82	221	52.5	<i>F.S.</i>	63	8'30"	68	149	44.2
<i>B.B.</i>	46	10'30"	86	319	41.7	<i>D.K.</i>	34	9'00"	89	165	39.2
Mean.....	33.2	10'18"	80.8	310.6	46.1		33.3	9'30"	67.1	178.6	45.7

In certain instances extreme changes in the clot resistance are paralleled by changes in the clot volume. In polycythemia vera the percentage clot volume is markedly reduced below normal. In 10 cases the mean percentage clot volume was 23.7 as compared with the normal of 46 (table 3). This is associated with a marked increase in the clot resistance above normal.

On the other hand, thrombocytopenic purpura is accompanied by a marked reduction in clot resistance and a marked increase in clot volume. The mean clot resistance of 4 untreated cases of typical thrombocytopenic purpura was 19 ohms.

The mean percentage clot volume was 67.5 as compared with 46 in the normal. These observations indicate that the degree and strength of clot retraction has an important bearing on the clot resistance. Further studies of this nature will be reported later.

The Primary Peak, a Measure of the Onset of Clot Retraction. Early in the studies it was noted that a plateau or peak followed by a dip sometimes occurred, terminating the primary rise. This dip was of inconstant occurrence and proportions. Further

TABLE 2. CLOT RESISTANCE AND THE FORMED BLOOD ELEMENTS

EX- PERI- MENT NO.	SEX	OHMS	PLTS.	RBC (MIL)	WBC	CLOT VOL. %	EX- PERI- MENT NO.	SEX	OHMS	PLTS.	RBC (MIL)	WBC	CLOT VOL. %
180	F	977	264,000	6.72			263	M	196	599,760	4.76	12,700	44.2
110	M	908	742,800	5.45	111,300		415	F	195	2,794,000	3.40	9,900	32.5
132	M	899	438,000	6.09	8,000	23.3	125	F	192	224,000	4.77		41.7
114	M	852	584,000	7.30	17,000		102	F	188	518,000	3.81	8,750	
90	F	729	574,000	9.18	19,500		93	M	174	688,000	3.45	124,000	
95	M	613	280,000	9.34	10,250		115	F	174	276,000	4.81		
216	M	598	378,000	8.40	9,500	52.5	153	F	172	294,000	4.20		50.0
452	M	573	323,000	3.23	51,700	35.0	147	M	171	106,000	4.39	3,100	52.5
244	F	553	750,000			33.3	108	F	132	280,000	4.00		
448	M	514	557,000	5.45	23,200	40.0	259	F	131	1,200,000	3.12	20,800	39.2
194	M	433	272,680	4.00	119,000	20.0	96	M	130	323,000	4.05	5,800	
185	F	417	202,000	5.22	14,900		97	M	128	170,000	2.94	11,800	
118	F	365	210,000	4.21	11,000		113	F	126	196,000	4.56	5,100	45.8
122	F	359	536,000	4.43	13,000		262	M	125	189,000	4.50	4,000	
399	M	353	180,000	5.55	14,000	34.2	99	M	119	275,000	3.56	8,000	27.5
107	M	331	391,000	5.43	7,800		123	M	115	1,530,000	3.06	16,000	54.2
104	M	302	504,000	4.99			227	M	122	592,800	3.80	10,200	
100	M	299	398,000	4.82	8,100		103	F	114	397,000	3.61		
109	M	278	140,000	5.60	5,200		106	F	101	140,000	4.28		
411	F	276	416,000	5.20	8,000	50.0	350	M	96		3.51	27,900	33.3
121	F	216	243,000	4.50			191	F	63	164,220	3.22	5,900	41.7
246	F	197	1,733,760	3.87	46,000	33.3	289	M	7	26,260	2.54	14,200	41.7

study indicated that this was due to adventitious adhesion of the blood to the cell walls and consequent failure of the clot to retract normally around the electrodes. This peak, the primary peak (fig. 1), may be induced by causing adhesion of the blood clot to the cell walls and, hence, retraction of the clot away from the electrodes. This may be brought about by roughening the walls of the cells, by placing a fine screen around the inner walls of the cell or by similar treatment.

The primary peak may also be induced by placing the electrodes in contact with the walls of the cell. In this manner the retraction of the clot from the cell walls promptly exposes the electrodes to the highly conducting serum. Siliconing above the electrodes reduces the adhesion of the clot to them and interferes with the electrolytic conductivity only slightly.

Under both sets of conditions the clot resistance rises in characteristic manner until the primary peak is reached at which time it drops sharply. When the primary

peak is induced by roughening of the cell walls the E.R.C., depending on the strength of clot retraction, will often resume its upward trend. When the electrodes are placed at the cell walls the resistance continues downward until it approaches that of the serum with which the electrodes are in contact. The primary peak occurs before clot retraction is observed visually and never following it.

The appearance of the primary peak, when caused by adhesion of the blood clot to the cell walls, is related to the clot resistance. When the measured clot resistance is high in the neighborhood of 700 to 800 ohms, the primary peak occurs infrequently. When the clot resistance is in a middle range of about 300 to 500 ohms, the primary peak occurs fairly frequently; whereas in a lower range of resistance, below 300 ohms, the peak is almost always present. This is further evidence relating the clot resistance to the strength of clot retraction. While only a limited number of tests have as yet been performed with the second technique, it is likely that it will more consistently

TABLE 3. CLOT RESISTANCE AND CLOT VOLUME IN POLYCYTHEMIA VERA

SUBJ.	OHMS	CLOT VOL. %	SUBJ.	OHMS	CLOT VOL. %
<i>I.L.</i>	899	23.3	<i>R.G.</i> ¹	692	20.0
<i>M.N.</i>	895	20.8	<i>C.R.</i> ¹	650	15.8
<i>K.F.</i>	866	34.4	<i>A.T.</i> ¹	504	10.8
<i>C.R.I.</i>	840	36.3	<i>S.Br.</i> ¹	430	25.0
<i>M.T.</i>	738	15.0	<i>A.V.</i> ¹	238	35.8
Mean.....					23.7

¹ After therapy.

yield a primary peak. Since the primary peak is produced either by the adhesion of the clot to the cell walls with the first method or by the retraction of the clot away from the cell walls with the second method, it is probable that the primary peak is related to clot retraction and is an index of the onset of clot retraction.

The time of appearance of the primary peak with the first method was between 29 and 33 minutes after taking the blood. With the second method the primary peak occurred between 27 and 35 minutes after taking the blood. These observations are consistent with the visually observed clot retraction time of approximately 30 minutes. The peak is detectable within one minute, but the reliability of the technique has yet to be established. When the R.C.T. is subtracted from the primary peak time it is seen that clot retraction commences 19 to 23 minutes after the onset of fibrin formation.

The above data, although tentative, are sufficient to indicate that the primary peak is probably capable of yielding an objective and accurate measure of the onset of clot retraction.

DISCUSSION

The curve of resistance offered by the whole blood clot to the passage of current between platinum electrodes buried in its substance has a distinctive pattern over a

period of 40 to 48 hours. This curve, named the electrolytic resistance curve (E.R.C.), has certain characteristics which are of physiological significance.

The initial point of interest is the resistance clotting time (R.C.T.) which marks the time of onset of the rise in the E.R.C. It is associated with the initial precipitation of minute strands of fibrin upon the electrodes. Therefore, the R.C.T. would seem to determine the onset of clot formation. This is to be distinguished from those methods, such as the Lee-White, which determine the time required for a given volume of blood to clot solidly. In the light of the studies on the rate of prothrombin conversion and, hence, on the rate of fibrin formation, it would seem that the R.C.T., which measures the onset of fibrin formation, has a more specific physiological significance than those methods which measure clotting *en masse*.

Both the syringe with which the blood is obtained and the glass cell in which the blood is placed are coated with Dri-film no. 9987. This practice permits the maintenance of the fluidity of the blood for exceptionally long periods of time. The platinum electrodes introduced into the blood function as a reproducible stimulus to precipitate clotting; and the recording galvanometer furnishes an objective and highly precise indicator of the moment when the resistance starts to increase. Hence, the technique would seem to offer the most nearly ideal conditions available for the determination of a clotting time. It suffers from one serious defect, namely, that when there is no increase in resistance there is no R.C.T.

In the normal white male the R.C.T. is 10.3 ± 1.0 minutes; in the normal white female the R.C.T. is $9.5 \pm .94$ minutes. There is no statistically significant difference between these two times and the mean normal R.C.T. is 9.9 minutes. A statistically significant correlation appears to exist between the R.C.T. and the Lee-White clotting time.

During the first hour or two the resistance rises rapidly in what is termed the primary rise. In the following 20 hours there may be a smaller secondary rise during which the E. R. C. reaches its maximum and begins to decline. The maximum resistance measured from the resistance of the fluid blood as a baseline (i.e. from which the resistance of the fluid blood is subtracted) is named the clot resistance. The clot resistance itself does not appear to be related to the fluid blood resistance in any significant manner.

It is notable that the clot resistance in the normal white male and female is strikingly different, 311 and 179 ohms respectively. Thus the male clot resistance is 74 per cent greater than that of the female.

There are two conditions in which marked changes in the clot resistance are accompanied by changes in the percentage clot volume. In polycythemia vera the clot resistance is very large and the clot volume is extremely small. This is a constant and striking finding in this disease and the conclusion is inescapable that both the clot volume and the clot resistance are indications of a markedly increased strength of clot retraction.

On the other hand, in thrombocytopenic purpura a sharp reduction in clot resistance is paralleled by a large percentage clot volume, which is indicative of a poor clot retraction. Following splenectomy, the clot resistance and the clot volume both return to normal as the platelet count rises.

These observations suggest that the clot resistance is intimately related to the clot retractile function of the platelets. In the range of resistance below 300 ohms, a statistically significant correlation exists between the clot resistance and the platelet count. Unfortunately, sufficient data are not yet available in the higher ranges of resistance.

Since clot retraction functions in the production of the clot resistance advantage may be taken of this to measure the onset of clot retraction. By roughening the walls of the cell containing the blood so as to cause adhesion of the clot it is possible to interfere with the contraction of the clot about the electrodes. By placing the electrodes in contact with the walls of the cell clot retraction exposes the electrodes to the highly conductive serum. Both procedures will cause a sharply defined peak—the primary peak—of the clot resistance, which is a measure of the onset of clot retraction. In preliminary observations the onset of clot retraction occurred between 27 and 35 minutes after the taking of the blood and 19 to 23 minutes after the onset of clot formation (after the R.C.T.).

Thus the measurement of the clot resistance, which in its entirety is called the electrolytic resistance curve, yields three types of information which seem to have physiological significance; the resistance clotting time, an objective registration of the initial onset of fibrin precipitation in whole blood, the clot resistance which is strikingly different in the two sexes and is in part a consequence of clot retractile force, and the primary peak which may make possible the objective measurement of the onset of clot retraction.

SUMMARY AND CONCLUSIONS

The electrolytic resistance curve of the blood clot is the curve of resistance in ohms over a 40- to 48-hour period. The resistance clotting time, the onset of the rise in resistance, is associated with the initial precipitation of fibrin on the electrodes. It is, therefore, a measure of the onset of clot formation. The normal time in the adult human is 9.9 minutes.

The clot resistance is the maximum resistance of the electrolytic resistance curve measured from the resistance of the fluid blood as a base-line. It appears to be related to the strength of clot retraction in polycythemia vera and in thrombocytopenic purpura. In polycythemia vera a high clot resistance is found in association with a small percentage clot volume. This is apparently due to a greater strength of clot retraction. In thrombocytopenic purpura the reverse is true. The normal clot resistance is 311 ohms in the adult white male and 179 ohms in the adult white female. A statistically significant correlation exists between the clot resistance and the platelet count when the clot resistance was 300 ohms or lower.

The onset of clot retraction is measured by the primary peak which occurs, in preliminary studies, between 27 and 35 minutes after the blood is taken and 19 to 23 minutes after the onset of clot formation.

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FIBRIN, A FACTOR INFLUENCING THE CONSUMPTION OF PROTHROMBIN IN COAGULATION¹

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THE prothrombin consumption test as originally described (1) determines the prothrombin activity remaining in serum one hour after coagulation. By means of this test, Quick established that little prothrombin activity is lost when either platelet-poor plasma or hemophilic blood clots. These results are satisfactorily explained by postulating that in hemophilia a marked deficiency of thromboplastinogen exists so that only a small amount of prothrombin can be converted to thrombin, while in thrombocytopenia, the platelet enzyme which is required for the activation of thromboplastinogen to thromboplastin is lacking; therefore little thromboplastin can become available and consequently little prothrombin is consumed. Quick, Shanberge and Stefanini (2) have shown that the speed of prothrombin consumption increases proportionately to progressive increments of platelets, but that the final amount of prothrombin converted approaches a constant. These findings are in accord with the concept that the platelet factor is an enzyme.

Since the prothrombin consumption test offers to become an important clinical test, further study was undertaken especially to find why at times erratic results were obtained. In the course of this investigation, an observation was made which promises to provide a new insight into the mechanism of thrombosis and hemostasis. It was found that fibrin is probably the primary agent responsible for the continuous removal of thrombin in the early stage of coagulation thereby blocking and delaying the basic chain reaction which is mediated through the labilizing action of thrombin on the platelets.

EXPERIMENTAL

The same reagents that are employed in the original one-stage method for determining prothrombin are required (3).

Tricalcium Phosphate-treated Plasma (Calcium Phosphate Plasma). In the determination of prothrombin in serum, fibrinogen must be supplied. This is most easily accomplished by means of oxalated plasma from which component A has been removed by means of tricalcium phosphate. This adsorbant was first introduced by Bordet and Delange (4) who concluded that it removed proserozyme, and yielded a plasma which was essentially a solution of fibrinogen. Nolf (5) in 1945 presented

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experiments which showed that prothrombin (serozyme) consisted of two substances, thrombozyme which is adsorbed by tricalcium phosphate and thrombogene which is not removed by this agent. Whether thrombogene is identical with the labile factor remains undetermined, but for practical purposes it suffices to state that tricalcium phosphate-treated plasma serves as a satisfactory agent in the determination of the residual prothrombin activity of serum.

The procedure is as follows: A measured volume of a suspension of 0.08 M tricalcium phosphate³ is transferred to a test tube, 1 cc. for every cc. of oxalated plasma to be treated. The tube is centrifuged to pack the gelatinous calcium phosphates and the water is poured off. The required volume of human oxalated plasma is added and the mixture thoroughly and repeatedly stirred with a small glass rod. After 5 minutes at room temperature, the tricalcium phosphate is removed by centrifugation. The treated plasma lacks the essential prothrombin factors and therefore fails to clot after the addition of calcium chloride and thromboplastin.

Determination of Prothrombin Activity in Serum. To a mixture of 0.1 cc. of calcium phosphate plasma, 0.1 cc. of thromboplastin and 0.1 cc. of 0.02 M calcium chloride is added 0.1 cc. of serum by blowing from a serological pipette. The time required for the formation of a clot is accurately determined with a stop watch. It is to be emphasized that the serum must be added last. The general technique is the same as that of the prothrombin time test. The test is carried out in a water bath at 37° C. The detection of the incipient clot is best accomplished by gently tilting the test tube while holding it towards a distant source of light and observing it from underneath. All tests should be carried out in clean pyrex test tubes (13 x 100 mm.).

When it was discovered that the separation of serum from the clot influenced the prothrombin consumption, a standard technique was devised. The test tube containing the clotted blood was put in an International Clinical centrifuge and spun at full speed for exactly one minute. An additional half minute was taken to stop the centrifuge. The serum was immediately analyzed for prothrombin activity, i.e. exactly one and one-half minutes after the beginning of centrifugation.

It was realized early in the development of the prothrombin consumption test that it lacked the high degree of accuracy of the prothrombin time of oxalated plasma since serum contains varying amounts of thrombin in addition to unconverted prothrombin which becomes activated during the prothrombin time test. In the beginning of this study, no means were available to evaluate quantitatively the additive effect of thrombin on the prothrombin time. It was for this reason that in the original test, the prothrombin was determined only after the blood had been coagulated one hour since in that period it was assumed that all the formed thrombin would be removed by inactivation through the natural antithrombin, albumin-X (6).

Later it was found that more significant results were obtained if a series of clotted specimens of blood were analyzed for prothrombin after varying periods of

³ The tricalcium phosphate is prepared by slowly adding with vigorous stirring 66.6 gm. of anhydrous calcium chloride dissolved in 1 liter of distilled water to an equal volume of trisodium phosphate containing 158 gm. of the anhydrous salt. After adjusting the pH to 7.0, the precipitate is repeatedly washed by decantation until only a slight trace of sodium chloride remains. The suspension is diluted to 2 liters with distilled water, making it 0.1M. From this stock suspension, a 0.008M preparation is made by diluting 8 cc. with 96 cc. of distilled water.

time. Specifically, four test tubes each containing 2 cc. of freshly drawn blood were placed in a water bath at 37° C. For normal blood the coagulation time was arbitrarily taken as 10 minutes, which is roughly the time required for 2 cc. of blood to clot at 37° C. At the end of exactly 15 minutes after coagulation, i.e. 25 minutes after the tube was placed in the water bath, the first test tube was centrifuged for one minute and the prothrombin determined immediately and after 15, 30 and 45 minutes. After 30 minutes the second tube was centrifuged and the prothrombin time determined immediately and after 15 and 30 minutes. At the end of 45 and 60 minutes after coagulation, tubes 3 and 4 respectively were centrifuged and the prothrombin determined.

Uncorrected Prothrombin Time in Clotted Blood. In table 1A the results on normal subjects are presented by selecting the findings of one typical subject and also a composite of results obtained on 20 healthy medical students. It will be observed that in normal individuals the prothrombin time of the serum of the first tube immediately following centrifugation averaged 8 seconds, but it lost prothrombin activity rapidly on standing. Even in the remaining tubes, the prothrombin activity of the serum immediately after centrifugation was always relatively high, but fell quickly as soon as the serum and clot were separated from intimate contact with each other.

In hemophilic blood the prothrombin time of the serum of all the tubes remained constant and centrifugation apparently had no effect, but curiously the prothrombin time of the serum was found to be invariably shorter than that of the oxalated plasma. In thrombocytopenia the prothrombin time of serum obtained after the blood had been completely coagulated and allowed to remain in the water bath for standard periods of 15, 30, 45 and 60 minutes, is, as in hemophilia, actually shorter than the 12 seconds of the oxalated plasma. Thrombocytopenic serums tend to show a gradual loss of prothrombin activity on standing.

It became increasingly more obvious as the study progressed that the speed of prothrombin consumption was strikingly affected by the separation of the serum from the clot whether brought about mechanically by centrifugation or spontaneously by clot retraction. To test whether exposure of serum might be the explanation, a comparison was made of the prothrombin consumption in a specimen of blood covered with paraffin oil with one exposed to air. The results in table 1B clearly show that such exposure has no effect on the rate or extent of prothrombin conversion.

Effect of the Intact Unretracted Clot on the Prothrombin Consumption Test. The evidence pointed clearly to the unretracted clot itself as the factor responsible for the delayed activation of prothrombin. Since Hirschboeck (7) had shown that no clot retraction occurs in collodion-coated vessels, the prothrombin consumption of blood coagulated in collodion-coated test tubes was studied. Surprising results were obtained. From a typical experiment presented in table 2, it can be seen that native platelet-rich plasma (obtained by the use of silicone-coated glassware) which clotted in 27 minutes showed no prothrombin consumption even after one hour following the formation of a solid clot. The serum was obtained by mechanically compressing the clot and therefore no time was lost in getting the first specimen of serum for analysis. Quickly following the expression of serum, a marked decrease of the pro-

thrombin time occurred which reached its nadir in $3\frac{1}{2}$ minutes and then rapidly increased. These findings have a simple explanation. Immediately on freeing the serum from the clot a sudden formation of thrombin occurred, which caused the shortening of the prothrombin time, but soon the consumption of prothrombin had progressed to

TABLE 1. EFFECTS OF CENTRIFUGING AND OF EXPOSURE TO AIR ON PROTHROMBIN CONSUMPTION TIME IN CLOTTED BLOOD

	TUBE	PROTHROMBIN TIME (UNCORRECTED) OF SERUM IN SECONDS			
		Time after formation of solid clot			
		15 min.	30 min.	45 min.	60 min.
<i>A. Effect of Centrifuging</i>					
Normal	1	7½ ¹	22	28	32
	2		15 ¹	30	35
	3			23 ¹	33
	4				21 ¹
Composite of 20 normals	1	8 ¹	24	31	31
	2		13 ¹	32	35
	3			18 ¹	32
	4				22 ¹
Hemophilia	1	9 ¹	9	9	9
	2		8½ ¹	9	9
	3			9 ¹	9
	4				9 ¹
Thrombocytopenia	1	8 ¹	9	9½	10½
	2		9 ¹	10	12
	3			8 ¹	10
	4				8 ¹
<i>B. Effect of Exposure to Air</i>					
Exposed to air	1	7 ¹	22	24	29
	2		15 ¹	37	44
	3			22 ¹	44
Covered with oil	1	7 ¹	22	24	31
	2		16 ¹	41	50
	3			25 ¹	44

¹ Immediately after centrifugation.

such an extent that the prothrombin time of the serum increased. From this it became evident that little prothrombin was converted as long as the serum was in intimate contact with the fibrin clot, but that immediately after this contact was lost, a rapid disappearance of prothrombin occurred. To test this further the experiment was repeated in glass test tubes (table 3). Again the change in the prothrombin time immediately following the separation of serum from the clot was striking. Unfortunately, the prothrombin time of serum could not be interpreted in terms of true

prothrombin activity since the influence of the thrombin was not only indeterminable but obviously also was constantly changing since it was continuously being neutralized by the normal antithrombin, i.e. by the albumin-X.

Prompt Arrest of Prothrombin Conversion by Sodium Citrate. It was felt that further progress was contingent upon finding a means to separate the activity of thrombin already present in the serum from the thrombin formed from prothrombin during the prothrombin time test. The possibility that this might be accomplished by an anticoagulant such as sodium oxalate or sodium citrate was investigated. The following experiment was devised. Three test tubes each containing 2 cc. of blood from the same subject were placed in a water bath at 37° C. and allowed to clot. The clotting time was empirically fixed at 10 minutes, and 15 minutes more was allowed for the reaction to go to completion. At the end of 25 minutes, 0.2 cc. of 0.1M sodium oxalate was added to the first tube, 0.2 cc. of 0.2M sodium citrate to the second, and nothing to the third. The tubes were centrifuged for exactly one minute and an additional half-minute was required to stop the centrifuge. The prothrombin time was immediately determined. For the oxalate and citrate plasmas,

TABLE 2. PROTHROMBIN CONSUMPTION TIME FOLLOWING THE SEPARATION OF SERUM FROM CLOT OF PLATELET-RICH PLASMA¹ COAGULATED IN A COLLODION-COATED TEST TUBE

Time after separation of serum from clot ² in minutes.....	0	2	3½	5	12	15
Prothrombin time (uncorrected) of serum in seconds.....	12	11	10	12	15	16

¹ Plasma was obtained from blood by employing silicone-coated glassware. ² The plasma began to coagulate in 21 minutes and a rigid clot was formed in 27 minutes. One hour after coagulation appeared complete, the clot was compressed with a stirring rod to obtain serum.

0.2 cc. of 0.02M CaCl₂ was used. A typical result obtained with this procedure is presented in table 4.

From the findings obtained, it is clear that the objective was attained with sodium citrate. Thus, the prothrombin time of the serum to which this agent was added before centrifuging was 11 seconds and remained constant whereas the oxalated specimen was 10 seconds and the untreated blood was 6 seconds. In the latter two the prothrombin dropped, whereas it remained constant in the blood citrated before it was centrifuged. A logical explanation is that prior to centrifuging only a trace of prothrombin is changed to thrombin, but immediately on centrifuging a chain reaction is set in motion and large amounts of thrombin are quickly formed which accounts for the prothrombin time of 6 seconds. This could easily be mistaken for hyperprothrombinemia, but is merely a summation of accumulated thrombin plus thrombin formed during the prothrombin time test. When citrate is added before centrifuging, the chain reaction is not begun and the prothrombin time measures the prothrombin activity remaining in the clotted blood. The oxalated blood shows a temporary shortening followed by an increase and then becomes constant. Sodium oxalate does not stop coagulation instantaneously as Quick and Stefanini (8, 9) have repeatedly emphasized. Therefore, a certain amount of thrombin with an equivalent decrease in prothrombin occurs in the short period required for the oxalate to become completely effective. The prothrombin time of the oxalated sample

obtained immediately after centrifugation is a composite of the thrombin already formed and the quantity produced by excess thromboplastin. On standing the free thrombin is neutralized and when this occurs the prothrombin time measures the unchanged prothrombin remaining in serum.

The procedure of stopping the prothrombin conversion instantaneously with sodium citrate, then allowing the thrombin to become neutralized and analyzing the serum for unaltered prothrombin by the prothrombin time test, permits one to follow the progressive reaction occurring in blood clotted in a test tube. The actual procedure consisted in placing 2 cc. of blood in a series of test tubes and allowing them to remain in a water bath at 37° C. for 25 minutes. All the tubes were then centrifuged one minute and an additional half minute taken for stopping the centrifuge. At specific intervals of time, 0.2 cc. of 0.2M sodium citrate was added to each of the tubes consecutively. After 5 minutes, the prothrombin time of the citrated serum was determined. The prothrombin values were calculated from Quick's prothrombin curve. The striking results showing the insignificant consumption of prothrombin

TABLE 3. SPEED OF PROTHROMBIN CONSUMPTION (UNCORRECTED) OF CLOTTED BLOOD IN A GLASS TUBE FOLLOWING CENTRIFUGATION

Time after centrifugation in minutes.....	0	1	2	3	4	5	6	7	10
Prothrombin time (uncorrected) of serum in seconds.....	8	5	7	8	12	15	18	22	25

prior to centrifugation and the precipitous drop after the separation of free serum is given in figure 1.

DISCUSSION

It has always been tacitly accepted that when normal blood clotted in a test tube, the process was completed promptly, i.e. the fibrinogen was entirely converted to fibrin, nearly all the prothrombin became activated and the excess thrombin was mostly neutralized by the natural antithrombin, albumin-X. The results obtained in this study lead to a radically different view. Actually, so little prothrombin is changed to thrombin even 15 minutes or longer after a solid clot has formed that it is hardly measurable. It would be difficult to explain this observation by any of the older hypotheses of blood coagulation including the classical theory of Morawitz. It can, however, be readily accounted for by the new concept that a chain reaction occurs in which thrombin labilizes platelets which in turn liberate more enzyme to activate thromboplastinogen, thus accelerating the formation of more thrombin.

In the ordinary clotting of blood in a test tube, enough platelets disintegrate to activate some thromboplastin and therefore a small amount of thrombin is formed. It is quickly combined with fibrinogen for which it has a great avidity. The union is probably very transient as is the combination of an enzyme with its substrate. Very quickly a meshwork of fibrin is formed with an enormous adsorptive surface which immediately begins to remove the nascent thrombin so expeditiously and completely that little remains available for further labilization of platelets. This adsorption continues until saturation occurs, after which thrombin begins to accumulate with the result that labilization of platelets again resumes; the block to

the autocatalytic reaction is thereby removed and rapid consumption of prothrombin ensues. It is interesting that the adsorption of thrombin by fibrin has long been recognized. Howell (10) actually prepared his thrombin by extracting fibrin with 8 per cent sodium chloride solution and recently Seegers (11) has made quantitative studies of the adsorption of thrombin by fibrin.

When clot retraction takes place, the serum is no longer dispersed uniformly through the fibrin reticulum, consequently the rapid or almost instantaneous adsorption of thrombin is stopped. The autocatalytic reaction therefore is set up which explains why a rapid fall in prothrombin occurs promptly following the extrusion of serum.

The adsorption of thrombin by fibrin although clearly recognized has been entirely overlooked as a significant factor affecting coagulation and hemostasis. It has always been an enigma why the thrombosis in an area of injured tissue remains localized. Since it is well recognized that one part of thrombin can coagulate several thousand parts of fibrinogen, the potential thrombin contained in a few cc.

TABLE 4. ADDITION OF SODIUM OXALATE AND SODIUM CITRATE TO CLOTTED BLOOD PRIOR TO CENTRIFUGATION TO PREVENT CONVERSION OF PROTHROMBIN TO THROMBIN AFTER THE SEPARATION OF THE SERUM FROM THE CLOT WITH THE OBJECTIVE OF OBTAINING A TRUE VALUE OF PROTHROMBIN CONSUMPTION

Time after formation of solid clot	Prothrombin Consumption Time		
	15 min.	30 min.	60 min.
Normal blood + sodium oxalate 0.1M ¹	10	13½	13½
Normal blood + sodium citrate 0.2M ¹	1.4	11	11
Normal blood.....	6	26	31

¹ 0.2 cc. added to the 2 cc. of clotted blood. ² The prothrombin time of oxalated plasma was 11½ seconds.

of blood is enough to cause generalized thrombosis of the complete vascular system. The physiological safeguards have never been clearly defined. The existence of a plasma or serum antithrombin (albumin-X) has been generally accepted, but this agent by no means acts instantaneously; therefore it is improbable that it alone could block the chain reaction from being set into motion. In fact there is experimental evidence that it cannot. One of us (A. J. Q.) had the opportunity through the courtesy of Dr. F. H. L. Taylor and his associates at the Boston City Hospital to follow the prothrombin consumption in a child with congenital afibrinogenemia. In 5 minutes after the native platelet-rich plasma had been transferred from a silicone-coated test tube to a plain glass tube, the chain reaction was in a high state of acceleration, thus showing that the albumin-X did not neutralize the thrombin sufficiently rapidly to impede the autocatalytic reaction.

In view of the findings presented it seems clear that the fibrin clot itself becomes physiologically the most important antithrombin. Its puts, so to speak, the brake on the autocatalytic reaction of coagulation and keeps it localized to the area in which vascular damage has occurred. Albumin-X only functions as a secondary defense since it removes or inactivates thrombin relatively slowly. Paradoxically, the fibrin clot which is feared most in thrombo-embolic diseases is perhaps the most im-

portant protection against extension of thromboses beyond the area that requires the thrombi to effect hemostasis.

From the above findings one may postulate that clot retraction *in vivo* may be distinctly dangerous since the extruded serum separated from the intimate contact of the adsorptive surface of fibrin will allow thrombin to form, which immediately causes an extension of intravascular clotting. If this assumption is correct the factors that favor clot retraction increase the danger of progressive intravascular clotting. One such factor would be anemia, for the smaller the cellular bulk, the faster and more complete is the clot retraction. The second factor is a rise in the number of platelets since clot retraction is roughly proportional to the platelet count.

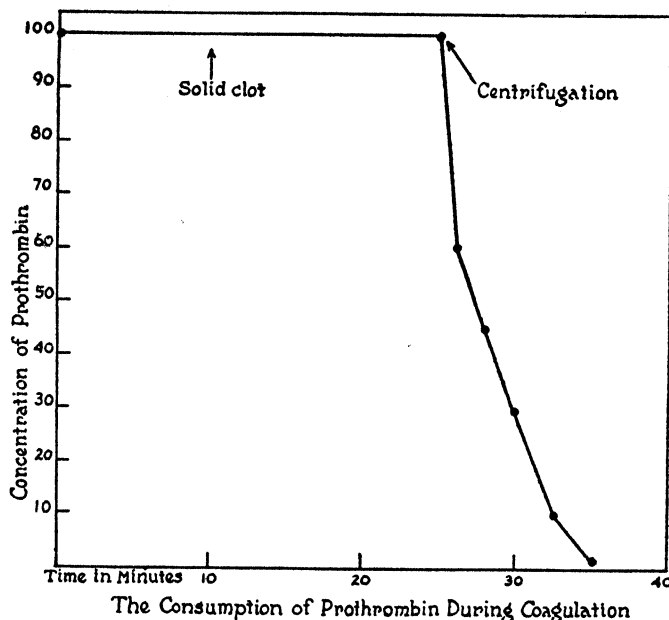


Fig. 1. PROTHROMBIN CONSUMPTION before and after the separation of the serum from the fibrin clot.

SUMMARY

When normal human blood is allowed to clot and to stand for one hour, more than 60 per cent of the prothrombin is usually consumed as measured by the prothrombin time of the serum. Serum obtained from clotted blood before retraction occurs has a strikingly short prothrombin time immediately after centrifugation, which quickly becomes prolonged, showing that a rapid decrease in prothrombin occurs following the separation of serum from the clot.

If sodium citrate is added to clotted blood prior to centrifugation, the serum obtained has a normal prothrombin time of 11 to 12 seconds, which indicates that the abnormally short prothrombin time of serum immediately after centrifugation is due to a summation of thrombin formed during centrifugation and the amount produced during the prothrombin time test.

The insignificant consumption of prothrombin in unretracted clotted blood is explained by the rapid and complete adsorption of the freshly formed thrombin by the fibrin clot with its large adsorptive surface that is in intimate contact with the dispersed serum. The continuous adsorption of thrombin prevents the initiation of the autocatalytic reaction of coagulation which is mediated through the labilizing action of thrombin on platelets. Fibrin appears therefore to be the most important physiological antithrombin. Its significance in hemostasis and thrombosis is discussed.

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BEHAVIOR OF THE LEUKOCYTES OF THE RABBIT DURING PERIODS OF TRANSIENT LEUKOPENIA VARIOUSLY INDUCED

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THE profound effects of injections into dogs of extracts of *Ascaris suum* and the fluid from hydatid cysts have been described in a previous report (1). The present study is concerned chiefly with observations of the behavior of the circulating leukocytes (as seen in transparent chambers inserted in the ears of rabbits) in response to intravenous injections of the parasitic materials just mentioned and a number of other substances.

It has been known since the work of Staub and associates (2) that intravenous injections of hepatic glycogen and injections of solution of acacia cause severe leukopenia of variable duration. Rocha e Silva and associates (3) have reported inhibition of anaphylaxis in the rabbit by injections of hepatic glycogen and a polysaccharide prepared from *Ascaris lumbricoides*. They also observed almost complete disappearance of platelets from the circulating blood. They found that the leukocytes disappeared only from unanesthetized rabbits or those anesthetized with dial and ether.

Hueper (4) reported that a solution of glycogen injected intravenously into dogs in single or repeated doses caused hematic reactions characteristic of the macromolecular hematic syndrome; namely, primary transitory leukopenia, secondary myeloid leukocytosis, anemia, accelerated erythrocytic sedimentation and increased clotting time.

With the aid of transparent chambers inserted into the ears of rabbits, using the well-known technic of the Clarks, Abell and Schenck (5) observed the behavior of the minute blood vessels and that of the leukocytes during anaphylactic reactions. It was found that in a properly sensitized subject an intravenous injection of horse serum or the introduction of horse serum into the moat of a special type of chamber was followed by a marked and characteristic response of the leukocytes, which expressed itself in an increased stickiness or adhesiveness of the cells. The cells adhered so tenaciously to the endothelium of the vessels and to each other that large clumps of leukocytes were formed. These aggregations of cells frequently were of sufficient size to interfere with the flow of blood through the vessels.

METHODS AND RESULTS

With the animals under anesthesia with pentobarbital sodium and by the use of sterile technic, transparent chambers were inserted into the ears of rabbits. When

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¹ Deceased. This work was begun with Dr. Graña while he was a Guggenheim Fellow working in our laboratory. The speculation and theoretical considerations must be attributed to the senior author.

the ingrowth of blood vessels had been completed, observations of the leukocytes were made with the aid of a compound microscope.

In a limited number of sensitized rabbits the interesting findings of Abell and Schenck already mentioned were seen by us after intravenous injections of horse serum.

The behavior of the leukocytes was also profoundly affected by intravenous injections of the following substances: 1) saline extracts of *Ascaris suum* in doses of 0.1 cc. for each kilogram of body weight; 2) 5 cc. of the fluid from hydatid cysts; 3) solutions of hepatic glycogen in doses of 200 mg. for each kilogram of body weight; 4) solutions of peptone; 5) 1 to 2 cc. of heparin solution (Connaught, 1000 units in each centimeter); 6) twice-normal concentrations of solutions of dried human plasma and of dried rabbit plasma; 7) 5 cc. of 6 per cent solution of acacia; 8) 1 to 5 cc. of a 6 per cent solution of a polysaccharide known as 'drolan' and also as 'dextran.'

The characteristic positive response usually began from 60 to 90 seconds after the intravenous injection of an appropriate dose of one of the effective substances.

Normally the leukocytes move haltingly along the margin of the blood stream, usually maintaining their spheroidal shape. A positive response to the substance injected is shown by a perceptible slowing. That they are becoming more adhesive is evidenced by some of them assuming an elongated or pear shape, particularly when the flow of blood through the vessel is quite rapid. When they have become sufficiently adhesive the leukocytes cease to be carried on by the most vigorous current and remain firmly attached to the endothelium. They also adhere to each other, forming large clumps that may become of such size as to interfere with the flow of blood through the vessel. It may be significant that the leukocytes adhere to the vascular endothelium and to each other but that the erythrocytes were never observed to stick to the leukocytes.

The most profound effect of all the substances used followed the injection of dextran. With most of the substances observed, the majority of the leukocytes remained out of the circulation for 15 to 20 minutes. However, injections of dextran immobilized them for 60 to 90 minutes.

On several occasions a continuous infusion of solution of acacia was given drop by drop for as long as 6 hours. The leukocytes adhered to the vessel walls while the solution was being administered, but 2 or 3 minutes after the infusion was stopped they resumed their movement.

An interesting result which is yet to be explained was obtained from experiments with dextran. Quite by accident it was discovered that after one injection of 5 cc. of dextran from which complete recovery had apparently occurred, another injection, whether immediate or long delayed, was without any observable effect. In a series of observations it was found that the leukocytes were sometimes unaffected by a second injection of dextran for as long as 4 days. In some of the experiments the leukocytes responded in a typical manner to injections of solution of acacia even though unresponsive to dextran, but in others they did not. As already indicated, the leukocytes did not show 'tachyphylaxis' when successive doses of solution of acacia were given.

From the work of previous investigators and a limited series of counts done in

our laboratory it has been shown that profound leukopenia follows the injection of glycogen and certain other substances. The leukopenia coincides with the period during which the leukocytes are observed to be adhering to the walls of the blood vessels (table 1). Consequently it is only an apparent leukopenia and is aptly termed 'transient.' An explanation of its temporary nature and an insight into its mechanism are rather clearly revealed by these findings. An explanation of why the cells become sticky has not been found.

In agreement with the reports of previous workers the number of leukocytes was greatly augmented as compared with control counts after their recovery from

TABLE 1. TYPICAL DATA ON THE NUMBER OF LEUKOCYTES IN THE CIRCULATION OF A SERIES OF RABBITS BEFORE AND AFTER AN INTRAVENOUS INJECTION OF A POLYSACCHARIDE, CALLED 'DEXTRAN'

RABBIT	DOSE OF DEXTRAN	TIME AFTER INJECTION	LEUKOCYTES PER CUBIC MILLIMETER OF BLOOD
1	cc. 1	Control 4 min. 60 min.	9,600 3,300 34,800
2	1	Control 6 min. 30 min.	13,200 8,400 24,400
3	3	Control 5 min. 30 min.	19,600 2,800 15,800
4	3	Control 8 min. 30 min.	9,600 3,000 15,800

adhesiveness and their return to the circulation. The leukocytosis is present for several hours.

Many other substances were injected without significant effect on the behavior of the leukocytes. This was true of injections of 1) 5 cc. of a 2 per cent solution of egg white; 2) 1 cc. for each kilogram of body weight of a 2 per cent solution of procaine hydrochloride; 3) as much as 10 cc. of a 5 per cent solution of purified gelatin in an isotonic solution of sodium chloride (Upjohn); 4) 5 to 10 cc. of isotonic saline solution, 5 cc. of 5 per cent glucose; 5) injections of various amounts of adrenal cortical extract; 6) 5 cc. of a normal concentration of human or of rabbit plasma; 7) 1 cc. of a normal concentration of horse serum in nonsensitized rabbits; and 8) 0.01 mg. of histamine for each kilogram of body weight.

COMMENT

It is obvious that the reactions of the leukocytes observed by Abell and Schenck during anaphylactic shock and those described in this report are fundamental in

nature. One can only speculate as to the process by which the leukocytes are made adhesive. The fact that so many different substances are able to elicit the stickiness of leukocytes emphasizes the nonspecific nature of the reaction. The transient leukopenia following injections of solutions of glycogen, acacia and such substances has given the impression that injection of substances of large molecular size elicits the phenomenon.

It has been suggested by Rocha e Silva and others that the vasculature of the liver and lungs removes the leukocytes and platelets from the circulation in anaphylaxis or after injections of glycogen, thus acting in a sense as filters. Our observations tend to support the concept that the leukocytes, after becoming adhesive, probably become attached to the endothelium in almost any region of the body and are not necessarily confined to the liver and lungs of the animal.

An insight may be gained from these observations into the mechanism involved in an inflammatory reaction. It is conceivable that an infectious process liberates leukotaxine (6) into the tissues, which finds its way into the capillaries and causes the leukocytes to become sticky and to adhere to the endothelium of the blood vessel. Eventually a large number of the cells would migrate from the blood vessel into the affected tissue with the characteristic leukocytic infiltration resulting. Abell and Schenck observed, during anaphylactic shock, the migration of many leukocytes from the vessels into the tissue spaces.

It has been found by most if not all observers that significant leukocytosis follows transient leukopenia induced by injections of such substances as glycogen. Why this should be is not at once apparent, but it might conceivably be the result of the effective operation of the mechanism that controls the number of leukocytes present in the circulating blood at any one time. Could it be that when the leukocytes become adherent to the vessel walls there is a call on the mechanism that controls the number of circulating leukocytes for more cells, which in turn would also become adherent, and thus a larger and larger number of leukocytes would be held out of circulation until released by the loss of their adhesiveness? Thereupon they would again return to the circulation in greatly augmented numbers, thus resulting in the leukocytosis so commonly observed.

SUMMARY AND CONCLUSIONS

Observations have been made of the behavior of the leukocytes as seen in the blood vessels that had grown into transparent chambers inserted into the ears of rabbits. The intravenous injection of extract of *Ascaris suum*, hydatid cyst fluid, solutions of acacia, glycogen and dextran, and many other substances caused the leukocytes to become sticky. They adhered to the vascular endothelium and to each other forming large clumps.

During the time when the leukocytes were adhesive, there was profound leukopenia. The duration of this phenomenon did not usually exceed 90 minutes. It may be concluded that the transient nature of the leukopenia observed in these experiments can be accounted for on the basis of the leukocytes being temporarily out of circulation as a result of their adhering to the vascular endothelium and to each other.

The possible relation of the adhesiveness of the leukocytes to certain inflammatory reactions is discussed.

The dextrone used in these experiments was kindly supplied by Dr. J. S. Lundy.

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AGE, BODY WEIGHT AND BLOOD HYPERTENSINOGEN

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GROSSMAN and Williams (1) reported that kidneys of young rats contained more renin than those of old rats. Using preparations free of hypertensinase and other nonspecific pressor substances, it has recently been shown, (2) that kidneys of young pre-pubertal rabbits contain more renin than those of older mature rabbits.

To continue investigations concerning age and the renal-humoral mechanism the following experiments were planned to study the hypertensinogen content of blood of large, old dogs and of small, very young dogs. Pressor responses to repeated injections of purified standard hog renin¹ were used as a test of the blood hypertensinogen, since it has been shown by Page *et al.* (3) that as plasma renin activator (hypertensinogen) is reduced or disappears, simultaneously the pressor response to injected renin is reduced or disappears.

PROCEDURE

The pressor responses to repeated injections of hog renin were tested in two groups of dogs. One group of 6 old animals varied in weight from 11 to 19.8 kg., and another group of 6 young animals, none older than 4 months, varied in weight from 3.2 to 5.0 kg. It was not possible to determine the exact ages of old dogs, but those selected had either lost or worn down most of their teeth and other signs of old age were apparent.

The dogs were given intraperitoneally 32.5 mg. of sodium pentobarbital per kilogram of body weight and blood pressure was recorded from the right common carotid artery with a mercury manometer. Blood pressure measurements were made 3 minutes after the renin injections, each of which consisted of 0.5 cc. containing 0.85 Goldblatt unit introduced briskly into the right femoral vein at 15-minute intervals until complete tachyphylaxis developed.

RESULTS AND DISCUSSION

In table 1 summarized blood pressure responses to the first two renin injections and the amounts of renin necessary to develop complete tachyphylaxis are presented. The blood pressure elevations from the first renin injection in the 6 old dogs varied from 6 to 12 mm. Hg and from 4 to 9 mm. Hg to the second injection. In this same group of dogs the amount of renin necessary to induce complete tachyphylaxis

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¹ Kindly supplied by Dr. O. M. Helmer, Lilly Laboratories for Clinical Research, Indianapolis, Ind.

varied from 5.95 to 11.05 Goldblatt units, that is, 0.40 to 0.85 Goldblatt unit per kg. of body weight.

Blood pressure elevations from the first renin injection in the 6 young dogs varied from 24 to 32 mm. Hg and from 14 to 18 mm. Hg to the second injection. In this group of dogs the amounts of renin necessary to induce complete tachyphylaxis varied from 17.0 to 26.35 Goldblatt units, that is, 3.74 to 8.2 Goldblatt units per kg. of body weight.

Since young pre-pubertal animals have a greater supply of renin it would seem that if this renin were to function to any important degree during pubertal cardiovascular adjustments, the supply of blood hypertensinogen available should be greater also.

TABLE 1. BLOOD PRESSURE REPOSSES AND AMOUNTS OF RENIN NECESSARY TO DEVELOP COMPLETE TACHYPHYLAXIS

DOG	WEIGHT	BLOOD PRESSURE RISE		GOLDBLATT DOG UNITS OF RENIN NECESSARY TO DEVELOP COMPLETE TACHYPHYLAXIS	
		First renin injection	Second renin injection	Total	Per kg. of body wt.
	kg.	mm. Hg	mm. Hg		
1	19.8	10	6	8.5	0.43
2	18.5	9	7	11.05	0.60
3	15.0	8	6	5.95	0.40
4	14.0	12	9	7.65	0.55
5	11.0	8	4	9.35	0.85
6	11.0	6	4	8.50	0.77
7	5.0	28	14	18.7	3.74
8	4.2	32	18	17.0	4.0
9	3.8	24	16	22.1	5.8
10	3.5	29	18	17.85	5.1
11	3.4	25	14	15.3	4.5
12	3.2	27	15	26.35	8.2

Although the body mass served could be a factor determining the magnitude of pressor responses from renin in the experiments just reported, one fact alone, namely that the 6 young dogs required a greater amount of injected renin to develop complete tachyphylaxis, indicates that young, small dogs have a greater available supply of blood hypertensinogen than larger, old dogs.

SUMMARY

Using pressor responses to repeated injections of standard hog renin as a test, the small, young dogs studied had a greater supply of available blood hypertensinogen than large, old dogs. A possible explanation is discussed.

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MECHANISM OF THE VASCULAR ACTION OF TETRAETHYLAMMONIUM CHLORIDE¹

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BURN and Dale (1) some 25 years ago showed that several quaternary ammonium compounds block transmission of impulses at autonomic ganglia. Recently, tetraethylammonium chloride² (abbreviated T.E.A.) was introduced after a broad study of its pharmacology by Acheson and Moe (2) as a blocking agent which is comparatively safe, even in human beings. Since then, wide interest has been aroused, especially by Lyons and Hoobler, in the possible application of this drug, either as a test of autonomic activity or as a therapeutic chemical sympathectomy.

Acheson and Moe (2) had shown in cats and dogs that doses of from 0.1 to 10 mg/kg. body weight reduce arterial pressure, usually with diminished heart rate, the magnitude of the fall being a function of the dose. The fall was not due to action on the heart, the vascular smooth muscle, or the medullary vasomotor center, but rather from a block in the ganglia of efferent pathways of the sympathetic vasoconstrictor nerves. Further, they showed when the dose was large enough, the response was pressor, probably accompanied by discharge of adrenalin. Intra-arterial injection usually produced no change in blood flow, though occasionally decreased it without change in systemic pressure, indicating vasoconstriction.

Our interest in its relationship to hypertension arose when we found that tachyphylaxis to renin could, under certain circumstances, be overcome by large doses of the drug (3). Further, it was shown that the action of many other vasoactive drugs was greatly augmented (4, 5). During the course of these investigations, a number of other observations were made on the action of the drug itself, which aid, we hope, in understanding at least from our point of view its greatest failure, namely to select hypertensive patients for lumbodorsal sympathectomy (6-8).

METHOD

Except for hepatectomized dogs or those paralyzed by spinal cord section, the experiments were performed under pentobarbital anesthesia. Aseptic technique was employed for the operations with occasional exception. The animals were carefully

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² Etamon Parke, Davis and Co. We are indebted to Dr. E. C. VonderHeide for part of the drug used in this investigation.

nursed after operation, especially after destruction of parts of the nervous system. This treatment included *a*) frequent turning, *b*) emptying the bladder, *c*) fluid administration, *d*) keeping the dog clean, warm and dry, and *e*) administration of penicillin if necessary.

Methods used for determining augmentation of a variety of test drugs have already been described (9). In most of the earlier experiments, single doses of 10 mg/kg. of T.E.A. were given into the femoral vein. Later the dose was reduced to 5 mg/kg. because in some animals, as for example after hepatectomy, the larger dose usually was fatal. After cord destruction, the carotid sinus mechanism was found excessively active to such drugs as nor-adrenalin and to mechanical stimulation. Blood pressure was moderately low and the heart rate very slow. Attempts at denervating the sinus usually resulted in stoppage of the heart unless the vagi were cut first. Many animals were saved by the simple procedure of vagus section followed, rather than preceded, by exclusion of the carotid sinuses.

When inactivation of the carotid sinus mechanism is referred to it means tying off above and below the bifurcation of the carotid artery and section of both vagus nerves.

The drugs used for testing vascular responsiveness were adrenalin 0.0025 mg., nor-adrenalin³ (DL arterenol, 0.05 mg.), barium chloride 0.25 cc. of solution containing 18 mg/cc., renin (0.1 cc. elevates pressure 25 to 35 min. Hg) and angiotonin 5 cat units as defined by Plentl and Page (16).

RESULTS

Effect of T.E.A. on Arterial Blood Pressure. Regardless of whether pentobarbital anesthesia is used or not, the response of the blood pressure to intravenous T.E.A. (5-10 mg/kg.) is variable. The usual pattern consists of a slight rise followed by a prolonged fall, as shown by Acheson and Moe (2). The second dose augments the rise and reduces the fall and the third usually elicits a pure rise. The response is converted from depressor to pure pressor by giving repeated injections of the drug.

Tests on 94 normal anesthetized dogs showed such a wide range of response that an average figure would be almost wholly misleading. Some of these supposedly normal animals gave a pressor response after the first injection of T.E.A. without any depressor action. They responded as do animals deprived of their nervous system. Even in the same animal tested on different days, the response changes. For example, the response of one of 12 dogs studied repeatedly was -48 mm. Hg on Feb. 10, 1947; +28 mm., Feb. 20; -42, March 25; and +38, Sept. 9. This wide variability is not exceptional. Thus experiments in dogs in which a 'floor' or standard depression of arterial pressure resulting from T.E.A. is required need to be interpreted with unusual care because of this great variability. This observation confirms the variable floor found in human beings (10).

Dogs with experimental renal hypertension produced by wrapping both kidneys in silk respond to T.E.A. in the same way as normal dogs. In 6 experiments we found no consistent change in response before and after inducing severe hypertension (180-220 mmg. Hg) in the same animal. The pressure drop was the same in mm. Hg

³ Dr. M. L. Tainter of Winthrop-Stearns was kind enough to supply this material.

before and after eliciting hypertension rather than the pressure reaching the same level. Since the results of this part of the investigation are negative, they are not included in this report.

Hypotension and the Action of T.E.A. It has been observed repeatedly that when blood pressure falls spontaneously (30-50 mm. Hg) during the course of the experiment, or directly after severe surgical operations, even though recovery of arterial pressure occurs, response to T.E.A. and to test drugs falls to low levels. Under such circumstances, administration of T.E.A. often has little effect on blood pressure, and does not increase the action of the test drugs conspicuously. Administration of large quantities of saline aids in restoring blood pressure more nearly to normal, when augmentation may appear following injection of T.E.A.

Many experiments show that the refractoriness following hypotension is not a direct function of the lowering of blood pressure. It not unusually occurs at hypertensive levels and is not immediately, if at all, relieved by T.E.A. Time, administration of fluids and maintenance of normal blood pressure levels are the factors we find important in overcoming this refractory state. After a prolonged period of hypotension and refractoriness, during which repeated injections of T.E.A. give no significant augmentation, spontaneous rise in arterial pressure usually heralds a return of responsiveness to higher levels.

Effect of Anterior Rhizotomy. Five successful experiments were completed in which anterior rhizotomy was performed by Dr. Robert Taylor either by one operation or in stages. The animals were allowed time for recovery. When the roots from C₆ to L₄ were included, the responses to the test drugs were uniformly increased over pre-operative tests. Administration of T.E.A. produced still further augmentation. The initial and subsequent responses to T.E.A. were pressor.

If the rhizotomy extended from D₇ to L₂ only, the initial pressor response to T.E.A. was exaggerated but the subsequent depressor component was powerful. Thus, in one dog, after this operation, T.E.A. caused a rise of 34 followed by a fall of 40 mm. Hg. But 6 days later, after extending the rhizotomy to C₈, the response became purely pressor, 42 mm. Hg.

Anterior nerve root section from C₇ to L₃ produced augmentation but was inadequate as a means of replacing the full effects of T.E.A.

'Total' Surgical Lumbo-dorsal Sympathectomy. At different operations, the ganglia were removed from the stellate down to the fifth lumbar on both sides by Drs. Robert Taylor and Charles Devine. The left vagus nerve was sectioned two days after the right and the test made the next day. Augmentation of adrenalin response was especially prominent, while barium and renin showed little change. The response to T.E.A. was always pressor.

In a dog 20 days after completion of the sympathectomy, atropine was given to paralyze the vagus nerve endings before responsiveness was determined. Pentobarbital anesthesia was used. T.E.A., just as in the dogs with their cords destroyed, gave a pure pressor response, but augmentation was still produced though it was not great. In a third dog tested 8 days after completing the sympathectomy but without use of vagotomy or atropine, just as in the other dogs no initial depressor responses were observed. Each dose of T.E.A. exhibited only pressor action.

Thus, so-called 'total' surgical sympathectomy is an insufficient operation to

replace wholly the effects of T.E.A. in heightening the action of other drugs. It so alters the animal that depressor responses are not observed, being replaced by pressor ones.

Effect of Cord Destruction. In all of these nearly 100 experiments, except those in which the animal was to be killed, laminectomies and cord destruction were performed under aseptic conditions and the animals carefully nursed after the operation.

Fall in blood pressure could still be obtained on administering T.E.A. when the cord was destroyed from D₆ caudad, but above this level the response became more consistently pressor. Above D₁ no depressor responses and only pressor ones were observed as shown, for example, in table 1.

When a section of the cord was removed from C₇ to D₆ inclusive, and the animals tested 2 to 3 days later, the response was always pressor. The response to the test drugs was uniformly augmented as a result of the operation itself, but after T.E.A. it was further increased. For example, blood pressure was elevated 50 mm. Hg after

TABLE 1. EFFECT OF ACUTE PROGRESSIVE CORD DESTRUCTION ON VASCULAR RESPONSIVENESS

TABLE 1. EFFECT OF CORD PROBLEMS ON CORD DESTRUCTION									
SUBSTANCE INJECTED	TIME	ARTERIAL PRESSURE		RESPONSE	SUBSTANCE INJECTED	TIME	ARTERIAL PRESSURE		RESPONSE
		mm. Hg	mm. Hg				mm. Hg	mm. Hg	
Renin	2:26	164		24	<i>Cord ligated at C₆ and destroyed below</i>				
T.E.A., 100 mg.	2:33	168		-42	Nicotine	4:25	108		42
T.E.A., 100 mg.	2:36	134		6	Nicotine	4:31	106		44
Renin	2:38	140		50	Renin	4:34	108		62
<i>Cord ligated at D₆ and destroyed below</i>					T.E.A.	4:42	132		34
Nicotine	3:22	142		22	Nicotine	4:45	128		0
Renin	3:27	138		16	Renin	4:47	118		60
T.E.A.	3:57	128		+4-22	Nicotine	6:50	120		-18+12
Renin	4:00	116		48	Renin	6:55	118		56
					T.E.A.	7:18	134		32
					Renin	7:22	126		54
					T.E.A.	7:44	138		36

adrenalin in one such dog before T.E.A. and 68 mm. after, when the cord had been destroyed one half hour before. But the next day, the response before was 80 mm. Hg and after T.E.A., 144 mm. Hg. T.E.A. raised the pressure 84 and 74 mm. Hg respectively.

Removal of a 3 mm. section between C₅ and C₆, without destruction of the caudal portion of the cord one day before the experiment, greatly augmented the response to the test substances and T.E.A. only slightly increased it. The next day the distal cord was destroyed and assay made the following day. No significant change in responsiveness had occurred except for a return of a definite pressor response to nicotine. T.E.A. gave somewhat more augmentation than in the first experiment. In 5 other animals with the cord sectioned at C₆, the initial responses to the test drugs were surprisingly similar.

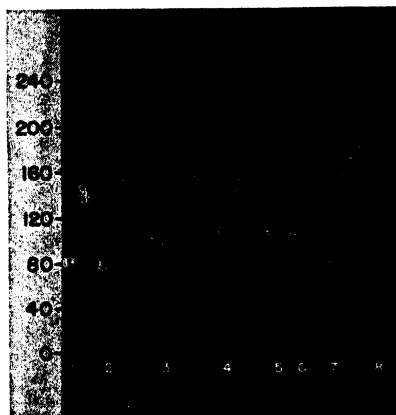
Cord Destruction from C₆ Caudal Followed by Inactivation of the Carotid Sinus Mechanism. Laminectomy and cord destruction from C₆ caudad two or more days before testing was performed on 88 animals. After the test, which was done without the necessity of anesthesia, the carotid sinuses and common carotid arteries were tied off and both vagus nerves cut (table 2).

Cord destruction alone augmented the responsiveness to the test drugs but administration of T.E.A. even further heightened the responses. Sinus exclusion and vagotomy were usually but not always followed by significant elevation of arterial pressure. Injection of T.E.A. always provoked a sharp pressor response in dogs with both cord and sinuses destroyed.

Pithing. Dogs under pentobarbital or other anesthesia were pithed by insertion of the pithing rod through the inner canthus of the eye. The results on vascular responsiveness were irregular. In some of the dogs the blood pressure fell to levels of from 30 to 60 mm. Hg and no augmentation to the test drugs occurred even after 6 hours. In others, responsiveness gradually increased apparently spontaneously and without benefit of T.E.A., while in still others, T.E.A. definitely aided in heightening the responses.

We assume this irregularity to be due to the different degrees of destruction of nerve tissue and of shock by the crude method used. Several years ago, we showed

Fig. 1. AUGMENTATION OF PRESSOR RESPONSE by T.E.A. in a dog with the spinal cord destroyed 2 days before from C₄ caudad. (1) Adrenalin, (2-4) renin, (5) T.E.A. 10 mg/kg., (6) adrenalin, (7-8) renin (no. 233). The response in a normal animal to the same amount of adrenalin is about +45 mm. Hg, renin +38 mm. Hg and T.E.A. +10-38 mm. Hg.



(11) that directly following pithing or other types of injury to the nervous system, refractoriness usually occurs for a short period.

Effect on Bilateral Nephrectomy. The kidneys were removed 2 days before the test in most of these experiments. Usually anesthesia was not used during the test unless the animal became unruly, when small amounts of pentobarbital were given intravenously. While the initial rise of blood pressure following T.E.A. injection seemed accentuated, the depressor component usually was present. For example, in one dog, the rise was 36 mm. Hg followed by a fall of 20 mm. Hg. The next dose raised the pressure 26 mm. followed by a fall of 4 mm. Hg. However, in many animals, only the pressor response was observed and repeated administration was accompanied by regular rises. Thirty complete experiments were performed. Augmentation of the test drugs occurred just as in normal animals.

Bilateral Adrenalectomy. Removal of both adrenal glands in normal dogs an hour or more before an experiment seemed to accentuate the depressor responses to T.E.A. and reduce the pressor ones. For example, in one of four such experiments, the successive doses of 5 mg/kg. of T.E.A. gave the following responses: +20 -30, +30

-10, +20 -2, +12 -16, +14, +12 -48, +18, +16 -6, +8 -14, +12 mm. Hg. The depressor response was almost constantly present despite repeated administration of the drug and the pressor was reduced.

The depressor response to T.E.A. was abolished by destruction of the cord from C₆ caudad, leaving the pressor response greatly heightened. Such preparations with

TABLE 2. RESPONSES IN MM. HG OF DOGS WITH CORD DESTROYED AND THE CAROTID SINUS MECHANISM INACTIVATED

	ADRENA- LIN	ADRENA- LIN	BARIUM CHLORIDE	RENIN	RENIN	T.E.A.	BLOOD PRESSURE
							<i>mm. Hg</i>
<i>No. 706</i>							
Control	48		44	36			88
After sinus inactivation	106		62	20		24	84
After T.E.A.	136		54	16		34	110
	140		52	48		18	102
	130			42		24	100
				58	50	8	108
			64	56	54		108
	138			48	40	40	100
	152		98	34	20		82
<i>No. 753</i>							
Control	44	54	24	62			120
After sinus inactivation	44	40	42	74			92
	84	28	18	32			68
	28	22	44				56
After 500 cc. saline	76	76		12			120
	92	86		14	24		100
				26		72	88
	94		86	14			78
<i>No. 743</i>							
2 days after cord destruction	20	28	22	36	34		112
Sinus inactivation	40		24	46			88
Next day	76		34	46	78		116
	70		36			24	128
				54		24	126
	82			40		36	116
				48	54	16	94
			36	30	52	18	92
	80			44			

the addition of bilateral nephrectomy and inactivation of the carotid sinus mechanism are therefore excellent ones to demonstrate the effects of the adrenal glands on T.E.A. response. Usually T.E.A. caused a sharp pressure elevation of 50 mm. Hg or more. But after adrenalectomy, it seemed somewhat reduced. In the different experiments, of which there were 7, the individual responses varied so much as to make strict comparison with other dogs with the adrenals intact lacking in significance. All that is safe to conclude is that in the absence of the adrenal glands, good

pressor responses are obtained on repeated administration of T.E.A. But the average response seems less than in similar preparations with intact adrenal glands. Sixteen experiments were completed.

Hepatectomy. The hepatectomies were performed by Dr. Ralph Prince, Dr. John J. Reinhard and Mr. William West. Several hundred such operations have now been performed. The result of this experience is that most of the dogs were in excellent condition when the testing was undertaken. We shall not at this time discuss the preparation and care of such animals despite its importance.

In these animals, injection of T.E.A. was followed by sharp fall in blood pressure and if the full dose of 10 mg/kg or even 5 mg/kg. was given, the result was usually fatal. Blood pressure fell to low levels and showed only weak tendency to recover. If doses of 2.5 mg/kg. were employed, sharp but not fatal falls occurred, which on repetition became progressively smaller. But significant pressor response did not occur, even though a total of 30 to 40 mg/kg. of T.E.A. was given. Rises of from 6 to 20 mm. Hg were observed in a few of the experiments. Thus, all of our results in the hepatectomized animal showed greatly increased sensitivity to the depressor effects of T.E.A. combined with almost complete loss of pressor effects.

Nephrectomy, Hepatectomy, Adrenalectomy. Since renin causes a rise in blood pressure when nephrectomy is performed just before hepatectomy (12) the possibility existed that nephrectomy might also influence the response to T.E.A. but this was not found true. Of 29 animals, 6 showed a rise of almost 20 mm. Hg when T.E.A. was administered. Adrenalectomy was then performed in 8 of these after nephrectomy-hepatectomy to determine whether this significantly affected the response. An example of this type of experiment is given in table 3. Augmented depressor effects of T.E.A. were observed as in the hepatectomized animals but possibly of somewhat greater magnitude. Pressor responses were never observed, even after repeated doses of the drug.

Spinal Cord Destruction, Carotid Sinus Inactivation, Nephrectomy and Hepatectomy. When the spinal cord was destroyed from C₆ caudad two days before the test and the carotid sinus mechanism inactivated the day of the test, the responses to T.E.A. were found to be sharply pressor with little or no depressor component. Removal of the kidneys and liver in such animals abolished the pressor response. Indeed, little response of any kind is observed to the injection of T.E.A., even though repeated doses of 5 mg/kg. were given. Occasionally, an animal exhibited a rise of 10 to 20 mm. Hg without subsequent fall.

Administration of Prisol⁴, Dibenamine⁴ and Benzodioxane to Dogs Subjected to Spinal Cord Destruction and Nephrectomy. Spinal cord destruction and nephrectomy were usually performed a day or two before the test. Anesthesia was not necessary after the operation because of the sensory paralysis. After pressor responses to adrenalin and nor-adrenalin were ascertained, 20 mg/kg. of dibenamine was given intravenously. Thirty minutes later the responses were again determined. It was usual to find the adrenalin response either biphasic with a large depressor component, or purely depressor; while the response to nor-adrenalin was reduced to about half. This

⁴ The Prisol was kindly furnished by Dr. Frederick Yonkman of Ciba Pharmaceutical Products, and Dibenamine by Dr. William M. Swain of Smith, Kline & French Laboratories.

confirms the observation of v. Euler (13) that nor-adrenalin is much more resistant to blocking than adrenalin. Prisol, 5 mg/kg., was then given and after a few minutes, the response was reduced from a control response of, for example, 120 mm. to 14 mm. Hg. Another dose of 2.5 to 5 mg. of Prisol blocked the nor-adrenalin response completely. Mixed solutions of Prisol (5 mg/kg.) and Benzodioxane (1 mg/kg.) were also used and found effectively to block nor-adrenalin.

TABLE 3. RESPONSE OF HEPATECTOMIZED, ADRENALECTOMIZED AND NEPHRECTOMIZED DOG¹

TIME, P.M.	B.P.	ADRENA- LIN	NOR- ADRENALIN	T.E.A., 5 MG.	T.E.A.	BARIUM CHLORIDE
1:15	112	16	22	-40		
2:44	32	16	38	-28		14
3:00	52			-18	-14	
3:43	56			-10		
3:48	54		72	0		
4:02	72			4	0	
4:20	70	66				Vagotomy
4:39	114		58			Angiotonin = 8 mm. Renin = 0.
4:57	Pithed					
5:05	82		62	0		30 Angiotonin = 8 mm. Tetramethylammonium, 10 mg. = 42 mm. Renin = 0 Angiotonin = 8 mm. Obstetrical pituitrin, 2 units = 72 mm. Angiotonin = 16 mm. Prostigmine, 0.25 mg. = 0. Ouabain, 0.25 mg. = 0. Ouabain, 0.5 mg. = 0. Sodium bicarbonate, saturated soln. 30 cc. 10.5 cc. adrenal cortex extract I.V. at 7:13 P.M. 10 cc. MgCl ₂ ·6H ₂ O 10 per cent I.V. at 7:55 Dose repeated
5:24	68	36	82			
5:52	80		90			
6:16	82	44	100			6
6:36	82	48	88			
6:52	82		86			
7:40	62		74			
8:02	64					
8:08			42			

¹ Dog 836.

Having blocked the already augmented responses to adrenalin and nor-adrenalin, T.E.A., which had previously given a striking pressor response (50-80 mm. Hg) now evoked no response or only small rises in blood pressure. As long as the response to nor-adrenalin occurred, T.E.A. could be expected to elevate blood pressure in spite of the completely reversed adrenalin response. There appeared to be a parallelism of pressor response to T.E.A. and nor-adrenalin (table 4). Nor-adrenalin, however, may produce a brisk response without necessarily a similarly good one being obtained from T.E.A.

Doses of Dibenamine and Prisol large enough to block the responses to nor-adrenalin usually reduced those to such peripherally acting substances as barium chloride and angiotonin.

Effect of T.E.A. on the Cat's Blood Pressure and Nictitating Membrane. Cats were anesthetized with pentobarbital, a carotid artery cannulated for blood pressure recording and the nictitating membrane attached to a writing lever. In some cases the membrane was sensitized by superior cervical ganglionectomy and in others by cocaine injection.

The initial 7 injections of 5 mg/kg., for example, produced a rise of 12 to 30 mm. Hg followed quickly by a fall of 38 mm. or more. The change to a pressor response came rather suddenly and was of the order of 44 mm. Hg. When the dose was doubled, rises of 120 mm. Hg occurred (figure 3). Responses to adrenalin, nor-adrenalin and barium chloride were greatly augmented.

During the period when arterial pressure fell as the result of T.E.A. injection, the nictitating membrane relaxed and continued to do so for some time after pure

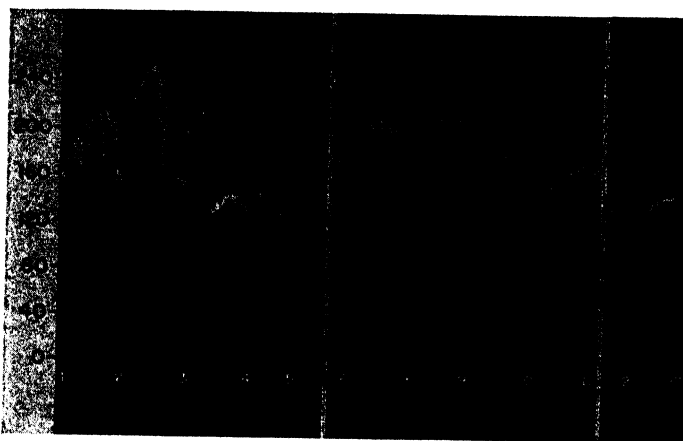


Fig. 2. PARALLEL RESPONSE of nor-adrenalin and T.E.A. 3 minutes after treatment of a nephrectomized dog with spinal cord destroyed ($C_6 \downarrow$) with Priscol and Dibenamine. (1) Nor-adrenalin, (2) T.E.A. 5 mg/kg., (3) nor-adrenalin, (4) T.E.A., (5-6) nor-adrenalin, (7) barium chloride, (8) renin, (9) T.E.A., (10) adrenalin, (11-12) nor-adrenalin, (13) T.E.A. (no. 833).

pressor responses were elicited. Then a change occurred and only contraction was observed of the same order as that resulting from injection of nor-adrenalin (fig. 3). For example, equipressor amounts of adrenalin, nor-adrenalin, T.E.A. and angiotonin caused the membrane to contract 15, 8, 5 and 10 mm.

No relaxation was observed after T.E.A. in the denervated membrane but contraction occurred when the depressor action on the blood pressure was over. Injection of 2.5 mg/kg. of Priscol abolished the response of the membrane to adrenalin, nor-adrenalin and T.E.A.

Pressor Response to T.E.A. in Patients with Cerebral Injury. Two patients, one dying of cerebral hemorrhage, the other with cerebral thrombosis, both exhibited pressor responses even on the initial injection of T.E.A. and without significant change in heart rate. As table 5 shows, some augmentation to barium chloride and angiotonin was observed. This was not great, probably due to the fact that the autonomic system had already been largely inactivated by the cerebral thrombosis. Thus, pressor responses to T.E.A. are observed in human beings as well as in dogs, after injury of the nervous system.

DISCUSSION

The evidence gathered by Acheson, Moe, Lyons, Hoobler and others strongly supports the view that the only significant action of T.E.A. is blocking transmission at ganglionic levels releasing the arteriolar tone due to sympathetic impulses. Blood pressure is lowered as a result and will continue to be lowered as long as there is continuing vasoconstrictor discharge from the spinal cord. With larger doses of T.E.A., the depressor action disappears, to be replaced by a pressor one.

In a few of our supposedly normal dogs, only pressor responses were obtained from the time injections were begun. Thus depressor responses, while being the usual initial ones, are not the only ones.

TABLE 4. EFFECT OF PRISCOL AND DIBENAMINE ON PRESSOR RESPONSE TO T.E.A. IN NEPHRECTOMIZED DOGS WITH SPINAL CORD DESTROYED

EXPER. NO.	ADRENALIN	NOR-ADRENALIN	T.E.A., 5 mg/kg.	DIBENAMINE, 50 mg.	PRISCOL, 5 mg/kg.	BLOOD PRESSURE, mm. Hg
8-30	84	116	36			100
				+12-14	36	110
	-12	22		+4-18	0	114
				38		110
		18	6			72
	-40	8	4			108
	-50	30	20			100
		3	18			114
		22	10			78
			100 mg.			
	8-33	112	106		14	128
	-24	44		8	6	120
		48		14	0	170
		32		26	0	160
		64	96			160
		72	34			124
		26	22			212
	+10-40	12	0			170
		22	4			150
	-46	30	12			124

If the autonomic ganglia are denervated or destroyed, by anterior rhizotomy, 'total' surgical sympathectomy or cord destruction, the depressor response fails to occur and is replaced wholly by a pressor one. It was one of the purposes of this paper to attempt the explanation of this interesting change, which is not unusual in the case of several other vasoactive drugs as well.

The phenomenon is not limited to dogs, for it occurred in two patients, one suffering from cerebral hemorrhage and the other from cerebral thrombosis. Neither of these patients showed any depressor response despite the fact that their arterial pressures were not low. Had they been excessively low, it might have been supposed that the cerebral lesion had abolished or diminished sympathetic vascular tone.

Since intra-arterial injection of T.E.A. causes no change in blood flow (2) there seems to be little or no direct action on arteriolar muscle to explain its pressor action. However, this observation must be qualified because Collins (14) found T.E.A.

capable of causing contraction of the isolated terminal ileum and, further, that it increases the responses to angiotonin and histamine. From the few measurements available, there appears to be insufficient change in cardiac output to account for the pressor effect. Some more indirect mechanism is thus suggested for which explanation must be sought.

Our results were for the most part obtained on dogs but some have been from a few cats. The same reversal of T.E.A. action was observed and in many cases more striking than in dogs. The nictitating membrane continued to show some relaxation well after pressor responses were obtained suggesting that the blood pressure is not the best indicator of complete autonomic blockade. Contraction of the membrane replaces relaxation very quickly and, in degree, more closely follows that produced by

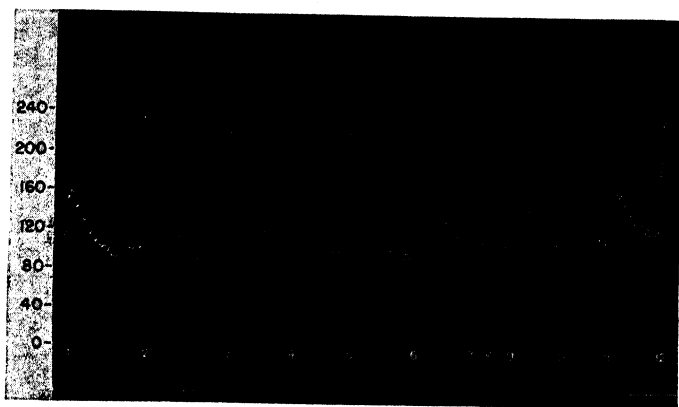


Fig. 3. EFFECT OF REPEATED DOSES OF T.E.A. on anesthetized (pentobarbital) cat's blood pressure and nictitating membrane (sensitized by cocaine). Eight injections of 5 mg/kg. body weight T.E.A. had been given over a period of 5 hours before. (1) T.E.A. 5 mg/kg., (2) nor-adrenalin 0.015 mg. (3) T.E.A. 10 mg/kg., (4) adrenalin 0.0025 mg. (5) nor-adrenalin, (6) T.E.A. 10 mg/kg., (7) saline 3 cc., (8) cocaine 2 mg., (9) adrenalin, (10) nor-adrenalin, (11) T.E.A., (12) angiotonin 6 units.

equipressor amounts of L-nor-adrenalin than adrenalin. It must be recognized that we are presently uncertain of the L-nor-adrenalin content of commercial adrenalin.

That the relaxation is of neurogenic origin follows from the fact that none occurs when the membrane is denervated. Contraction results, we believe, because of a humoral agent. Further, this agent is blocked by Priscol just as is adrenalin and nor-adrenalin.

Three facts about the pressor response to T.E.A. stand out clearly: 1), that it is blocked when the response to nor-adrenalin is blocked, but does not parallel blocking of the adrenalin response: 2), that it does not occur in liverless animals except occasionally and in minor degree (i.e., 20 mm. Hg). This suggests that the pressor response is due chiefly to liberation of a nor-adrenalin-like substance from the liver. The adrenal glands seem to contribute to a far less important degree. 3) In the case of the animals with cord and sinus mechanism destroyed, the augmentation of the action of adrenalin and nor-adrenalin which we have described previously (3, 4) is an additional factor.

The vascular response to T.E.A. might be thought of as follows: At first, blockade

of autonomic ganglionic transmission causes loss of vasoconstrictor tone in the blood vessels comparable to that following nervous system destruction, and blood pressure falls. Administration of more T.E.A. produces progressively less depression until only pressor effects occur. Since the depressor action is greatly increased in liverless animals, it may be supposed that a buffer mechanism has been removed with excision of the liver. Since the pressor response to T.E.A. roughly parallels the response to nor-adrenalin and is concurrently blocked by Dibenamine, Priscol and Benzodioxane, and since little or no pressor responses occur in absence of the liver, the buffer mechanism must comprise chiefly liberation of a nor-adrenalin like substance from the liver. This substance may well be hepatic sympathin-N, following the nomenclature of v. Euler. A small component of the rise may be attributed to adrenalin because removal of both adrenal glands appears to reduce slightly the rise when the liver is intact, or abolish altogether the slight residual rise to be expected from T.E.A. sometimes seen after hepatectomy or blockade with Priscol and Benzodioxane. Since the sensitivity of the vascular tree is greatly heightened to both nor-adrenalin

TABLE 5. PRESSOR RESPONSE AND TETRAETHYLAMMONIUM CHLORIDE AUGMENTATION IN A HUMAN BEING SUFFERING FROM CEREBRAL THROMBOSIS

INITIAL BLOOD PRESSURE mm. Hg	TEST SUBSTANCE	TIME	BLOOD PRESSURE RISE mm. Hg
84	Adrenalin, 1 ml. 1:1,000	10:51	42
92	Barium chloride, 45 mg.	10:57	28
102	Angiotonin, 18 units	11:05	22
88	Tetraethylammonium, 700 mg.	11:15	8
94	Adrenalin, 1 ml.	11:18	38
100	Barium chloride	11:23	52
124	Adrenalin, 1 ml.	11:40	40
137	Tetraethylammonium, 600 mg.	11:42	31
120	Angiotonin, 18 units	11:46	50

and adrenalin by T.E.A., that liberated endogenously would be expected to elicit greater responses than in untreated animals.

Our experiments show that vasomotor impulses of the kind blocked by T.E.A. have their outflow from below D₁; hence for routine studies, section of the cord at C₄ and destruction caudad is adequate. It was of especial interest that inactivation of the carotid sinus mechanism significantly heightened vascular responsiveness of the already highly sensitive animal. Administration of T.E.A. then caused insignificant further augmentation, although over a period of several hours the animals receiving T.E.A. were usually more sensitive than those not treated with it.

When the cord had been destroyed, the carotid sinus mechanism became much more conspicuous in its action. Its destruction in dogs with intact spinal cords produced little increase in responsiveness to vasoactive drugs, but with the cord destroyed heightening was pronounced. Further, during the early operations of carotid sinus destruction, most of our dogs died. When we realized that this was due to extreme vagus inhibition, and it was overcome, either by rapid cutting of the vagus nerves before sinus denervation, or by applying cocaine solution to the sinus, there were no further deaths from the procedure. The unopposed carotid sinus mechanism when stimulated becomes a lethal one.

The ability of the vascular system to adjust to severe injury and the loss of organs doubtless contribute significantly to its regulation. This was shown, for example, by the experiments in which the spinal cord was destroyed 10 days before and the carotid sinus mechanism inactivated immediately prior to the experiment, and then the kidneys, the adrenal glands and liver excised under ether anesthesia. These were not moribund animals, nor was anesthesia required after operation. Eight hours after such extensive surgery, the mercury manometer recording of blood pressure was indistinguishable from that of normal dogs. This is not to imply that the responses were the same. Rather, to be emphasized is the extraordinary power of the circulation to perform its primitive functions temporarily, at least, in the absence of vital organs. Reinhard, Glasser and Page (14) have shown that hepatectomized dogs with or without their kidneys withstand hypotension of long duration quite as well as more normal animals. It would seem unreasonable to suppose, however, that the vascular responses are identical.

Finally, the elucidation of the mechanism of T.E.A. action shows why the drug has not proved a satisfactory indicator of sympathetic activity. For instance, Birchall, *et al.* (6) found no correlation between the response to surgical sympathectomy in hypertensive patients and the chemical sympathectomy performed by T.E.A. If the response to T.E.A. has two components, the one depressing blood pressure the other elevating it, the resultant will always be a compromise between the magnitude of these forces. When most of the pressor factor is removed by hepatectomy-adrenalectomy, then a quarter of the usual dose produces severe unopposed fall in blood pressure. If the nervous factor (autonomic blockade) is abolished by previous destruction of the spinal cord, only a rise occurs. Thus, to determine the influence of the drug on ganglionic blockade alone on blood pressure, it is necessary to exclude an augmented humoral factor, having opposing effects to those of blockade.

Bilateral nephrectomy performed two days before the test is an example of a means of affecting the normal equilibrium between the two opposing factors. A common response in such animals to the initial T.E.A. dose is a rise in arterial pressure with little or no fall, as opposed to the usual fall in normal dogs. It might therefore be supposed that nephrectomy causes the retention of substances acting to reduce the vasomotor factor, leaving the humoral mechanism unopposed.

It should be noted that we were unable to detect any consistent change in the response to T.E.A. after renal hypertension was produced by wrapping the kidneys in silk. The fall of blood pressure in mm. Hg was roughly the same before and after hypertension. This is consistent with the demonstration by Freeman and Page (15) that the autonomic system does not exert a dominant influence in this particular type of hypertension.

SUMMARY

The blood pressure response of a large number of anesthetized dogs to intravenous injection of 5 or 10 mg/kg. body weight of tetraethylammonium chloride (T.E.A.) was irregular, ranging all the way from pure depressor to pressor, varying even from day to day in the same animal.

There was no consistent change in the response after the development of ex-

perimental renal hypertension by silk perinephritis. A spontaneous type of vascular refractoriness to a variety of vasoactive substances not infrequently occurred, often but not necessarily associated with a period of hypotension. Anterior rhizotomy from C₆ to L₄, 'total' surgical lumbodorsal sympathectomy, cord destruction from C₆ caudad or removal of section of cord between C₆ and C₆, all abolished the depressor component of T.E.A., leaving a pure pressor response. Inactivation of the carotid sinus mechanism in otherwise normal dogs caused little change in response but in dogs with the cord destroyed from C₆ caudad, the sinus mechanism was highly active. Its inactivation in cord-dogs augmented the pressor response elicited by T.E.A.

Bilateral nephrectomy increased the tendency for a pressor response to occur at the expense of the depressor. Bilateral adrenalectomy accentuated the depressor responses at the expense of the pressor. Hepatectomy greatly augmented depressor responses and all but abolished the pressor ones. If adrenalectomy was combined with hepatectomy and nephrectomy, no pressor response was observed. Removal of the liver and kidneys in animals with the cord destroyed and the sinus mechanism inactivated abolished the pressor responses to T.E.A. Administration of doses of Priscol, Dibenzamine and Benzodioxane, singly or combined, sufficient to reduce or block the response of arterenol also reduced or blocked the pressor response of T.E.A. Cats exhibited the same reversal from depressor to pressor response after injection of T.E.A. Relaxation of the nictitating membrane occurred regularly until well after the pressor response was established, then quickly reversed to contraction. The degree of contraction followed much more closely that due to equipressor amounts of nor-adrenalin than adrenalin or angiotonin. No relaxation occurred after the membrane was denervated but contraction was observed when the depressor action of T.E.A. was over. Injection of Priscol then abolished the contraction of the membrane to adrenalin, nor-adrenalin and T.E.A. Two patients with injury to the central nervous system have shown the initial pressor response to T.E.A. which occurs in dogs with parts of the nervous system removed.

CONCLUSION

These observations in dogs, cats and man suggest that the action of tetraethylammonium chloride (T.E.A.) on arterial pressure is compounded of at least three factors: 1) autonomic blockade reducing or eliminating tonic vasomotor impulses which lowers arterial pressure, 2) stimulation of the liver, to liberate a nor-adrenalin-like substance which, acting on a more than normally sensitive vascular tree, raises blood pressure, 3) less importantly, liberation of adrenalin from the adrenal glands. The net effect on arterial pressure is the resultant of the forces tending to lower opposed by those tending to elevate it.

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CARDIOVASCULAR EFFECTS OF LARGE VOLUMES OF ISOTONIC SALINE INFUSED INTRAVENOUSLY INTO DOGS FOLLOWING SEVERE HEMORRHAGE^{1,2}

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THE use of normal salt solution to restore a large blood deficit caused by hemorrhage has had few supporters. Harkins and McClure (1) in a review of the literature up to 1941 mentioned many investigations indicating that isotonic saline was the least beneficial of replacement fluids. In general, the arguments raised against saline were: *a*) that an isotonic crystalloid solution, having no colloid osmotic pressure, would be lost from the circulation too rapidly to exert more than temporary benefit, *b*) that loss into the tissue might even cause pulmonary edema (2), and *c*) that, where capillary permeability was increased, it might wash plasma proteins out of the blood (3) leaving the circulation in a worse state than before the infusion of the salt solution. There were, however, some supporters for the use of saline, namely Hoitink (4) and MacFee and Baldrige (5), the latter having success with the therapeutic use of large volumes of the fluid.

In March, 1942, the first of a series of articles by Rosenthal (6) appeared. Investigating the most effective treatment for burns and shock, produced in large numbers of mice, he found that oral, intravenous or intraperitoneal administration of isotonic (0.9 per cent) NaCl solution significantly reduced the mortality rate. Further experiments by Rosenthal (7-10) on tourniquet shock and hemorrhage indicated that transfusions with large volumes (8-15 per cent of the body weight) of isotonic saline were as effective in reducing mortality as transfusions with serum or whole blood. Fox (11) who tried large amounts of sodium lactate solution orally in severely burned patients reported favorable results. Sodium chloride solution is efficacious in preventing shock produced in dogs by venous occlusion according to Katz, Friedberg and Asher (12) who concluded from their experiments that the beneficial effect can be attributed to the sodium ion. Warren, Merrill and Stead (13), who studied the effects of saline infusion on tourniquet shock in dogs, felt that the hydration of the interstitial fluid compartment was the important factor in the maintenance of the blood volume. After removing the tourniquets they gave saline intravenously in large amounts and over long periods of time, and they reported that the plasma volume and arterial pressure were maintained at normal levels when the infusion was carried to the point of development of generalized edema. The fact is that the investigations in which isotonic saline was found to be beneficial are those in which the volumes given amount to two or more times the estimated blood volumes.

The purpose of the experiments reported in this paper has been to analyze the circulatory changes brought about by the infusion of large volumes of saline after severe hemorrhage and to ascertain to what extent the circulatory changes themselves may account for the favorable effects.

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PROCEDURE

Adult, mongrel dogs ranging in weight from 5 to 10 kg. were used. Each animal was offered unlimited water but no food for 24 hours before the start of the experiment. The dog was placed on his back on an animal board. The right and left femoral arteries and the right jugular vein were exposed under local procaine hydrochloride supported by Neothisol.³

The experiment consisted of a hemorrhage followed an hour later by a large intravenous infusion of isotonic saline. The clinical condition of the animal and especially the cardiovascular responses were observed for 24 hours after the bleeding. A dog living that long was considered a survivor. During the control period values for the heart rate, mean arterial pressure (arterial puncture, Hg manometer) and O₂ consumption were obtained, and blood samples were drawn for the estimation of plasma volume, plasma protein concentration, hematocrit readings and arterial and mixed venous O₂ content. The rectal temperature was taken only during the control period. Any dog showing evidence of fever was not used.

The hemorrhage was performed as described by Walcott (14) except that during the period following bleeding only one 7 cc. sample of blood was withdrawn. All of the above measurements, with the exception of the plasma volume, were repeated during the interval between bleeding and infusion as well as during the period following saline infusion. The saline used was a 0.9 per cent solution of chemically pure sodium chloride and distilled water freshly prepared for each experiment. It was warmed to 39° C. and injected through a catheter inserted in the right jugular vein. A rate of about 1.3 cc/min/kg. was empirically chosen from preliminary experiments (15). The administration of saline was stopped when the dog had received a volume equal to 15 per cent of the body weight. When the measurements were completed, one and a half to two hours later, the animal was removed from the board and placed in a metabolism cage. Water was given *ad libitum* but no food, and the measurements were repeated the following morning on all survivors.

Each dog was weighed before the experiment, at the end of the first day and before being put on the board the second morning. In most of the animals the bladder was emptied and washed out through a catheter immediately before starting the infusion; urine was collected during and following the administration of saline. The overnight urine loss as well as the amount of water drunk during the night were measured roughly on all except a few dogs.

The plasma volume was determined, using the dye T-1824 and the method described by Gregersen and Stewart (16). The dye was injected into the right jugular vein. For the control volume samples were drawn from the left jugular vein, while those for volumes measured on the following day were drawn from a femoral artery. The optical densities of the dye samples were determined with a König-Martens spectrophotometer (17). Semilog plots of the optical densities were used for calculating plasma volumes (18). Hematocrit values were obtained by centrifuging heparinized blood samples in Wintrobe tubes for 30 minutes at 3000 r.p.m. (radius 13 cm.). The total blood volume was calculated in the usual way (18a), using 0.96 as the correc-

³ Two parts of methyl methylene para amino phenylformate and 5 parts of hydroxybenzo-carbinol in refined almond oil. Caso Laboratories, New York City.

tion factor for the plasma trapped among the erythrocytes (19). The plasma protein concentration of each sample was measured with an Abbé refractometer (20).

The direct Fick method was used for determining the cardiac output. Arterial blood samples were drawn from a femoral artery into syringes rinsed with heparin solution. To obtain mixed venous blood a catheter, attached to a saline manometer, was inserted through the left jugular vein and manipulated into a position which, according to the criteria used, was considered to be in the right ventricle. The depth of the catheter, the characteristic impact of systole felt and seen along the tube and the manometric readings were the criteria used in judging the position of the catheter. For the determinations made during the control period and after the infusion the pressure readings were always between 20 and 10 cm. saline, and the systolic impact was firm and forceful. For the post-hemorrhagic determinations, however, the impact was feeble and the pressure was about zero. On the second day of the experiment the catheter's position was confirmed at autopsy and, in all except 3 animals, the tip of the catheter was found in the right ventricle. In one of these the tip of the tube was in the vena cava at the entrance to the auricle, and in the other two it was in the pulmonary artery. In order to avoid the presence of saline from the manometer in the blood samples the catheter was first flushed out by withdrawing 10 cc. into a separate syringe. After obtaining the mixed venous sample the 10 cc. of saline and blood were returned. Throughout the experiment the dripping of saline from the manometer's reservoir into the vascular system was prevented except during the control period when not more than 3 cc. passed through the catheter. Clamping off the catheter from the manometer after the former was filled with saline stopped the flow. This was sufficient to prevent clotting in the tube.

Arterial and mixed venous blood samples were analyzed for their respective O_2 contents by the Roughton and Scholander method (21). Analyses were made from 6 to 8 hours after obtaining the blood, the samples being stored as described by Roughton and Scholander. In agreement with these investigators, the precision of measuring O_2 content on the same blood sample was found to be ± 0.2 vol. per cent.

Oxygen consumption was measured by means of a Benedict-Roth spirometer. The dog breathed through a Blalock mask. Great care was taken in the application of the mask and in holding the dog's head during recording periods in order to avoid a leak around the muzzle. The concentration of O_2 within the spirometer was maintained at 35 ± 5 per cent by filling the gas chamber about one seventh with pure O_2 , the remainder being room air. Three minutes after starting to record O_2 consumption the first venous sample was drawn, followed 20 seconds later by an arterial blood sample. Without changing the arrangement in any way, another pair of samples was drawn two minutes later. The O_2 contents of the two arterial samples were averaged, as were the two venous O_2 contents, and the difference between the averages used in calculating the cardiac output.

RESULTS

Changes in Fluid Volumes, Hematocrit Values and Plasma Protein Concentration. Of the 27 dogs studied, 23 survived. The second day of each experiment the survivors were alert, responsive and walked and trotted when led from their cages.

Figure 1 shows the mean values of most of the fluid exchange data which were obtained from the 23 survivors. The average weight was 7.75 kg., the range extending from 4.8 to 11.3 kg. Urine outputs and water intakes though measured were not averaged because the range of variation was too great for the values to be significant. In spite of removing an average volume of 326 cc. of blood (42 cc/kg.) during the hemorrhage and of injecting an hour later an average of 1170 cc. of saline (151 cc/kg.), the mean weight increase at the end of the first day was only 0.25 kg. All dogs which

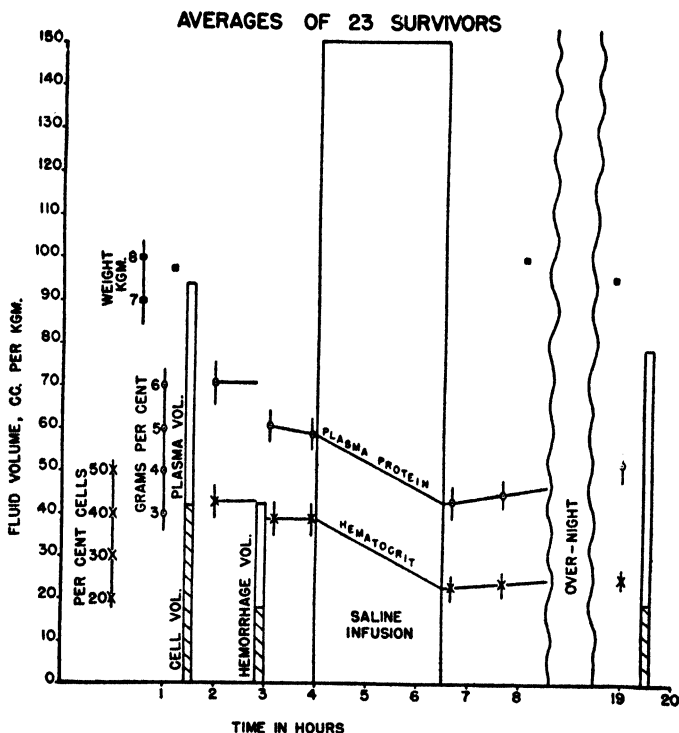


Fig. 1. EFFECT OF HEMORRHAGE and saline infusion on body weight, plasma volume, plasma protein concentration and hematocrit value in experiments on 23 surviving dogs. Average values are indicated by the characteristic points and average deviation is shown by the vertical line drawn through the point. Time values also represent averages.

survived showed a considerable diuresis which began within an hour after the start of the infusion. The rate of outflow increased until in some experiments more than 250 cc. was collected in the third hour. Even taking into account the urine output, the weight at the end of the day is lower than might be expected. The unmeasured loss of water from the lungs and skin would account for this. No diarrhea or vomiting was shown during the day by any of the dogs which survived. The diuresis continued throughout the night. When placed in his cage at the end of the first day each dog immediately drank several hundred cc. of water. During the night much more was ingested. Although the volume of fluid exchanged overnight varied considerably in different animals, the weight the next morning consistently showed a loss which averaged 0.2 kg. less than the average control weight.

The plasma and blood volumes, measured one hour before the hemorrhage, averaged 52 ± 4 cc/kg. and 94 ± 7 cc/kg., respectively. On the day after the infusion the plasma volume of every survivor, with the exception of one dog, showed an increase, the mean value reaching 60 ± 4 cc/kg. The red cell volume, however, was so greatly reduced by the bleeding that the average total blood volume measured the next day was only 79 ± 6 cc/kg.

The plasma protein concentration fell from an average control value of 6.1 ± 0.5 gm. per cent to 4.9 ± 0.4 gm. per cent one hour after the hemorrhage and after the saline infusion it was further reduced to 3.3 ± 0.4 gm. per cent. One would expect both of these changes, the first resulting from the compensatory dilution of the blood by the tissue fluids during and after hemorrhage, and the second resulting from the dilution of plasma by the saline infusion. The mean hematocrit values which are also listed in table 1 show the same relative changes. On the following day, however, the plasma protein concentration was found to have increased significantly but the hematocrit value remained about the same.

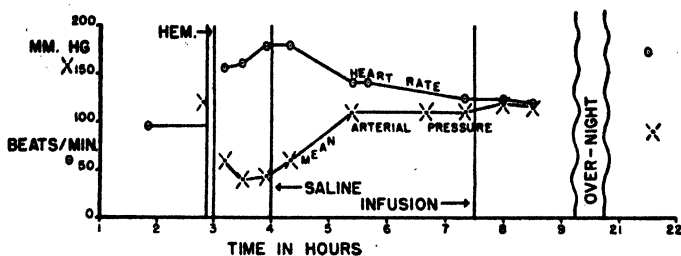


Fig. 2. HEART RATE AND BLOOD PRESSURE changes during a typical experiment; values of a single dog, no. 4.

Circulatory and O_2 Consumption Changes. The two curves in figure 2, representing typical fluctuations in blood pressure and heart rate, were taken from the protocol of experiment 4. After the hemorrhage, the mean arterial pressure fell from a control value of 120 mm. Hg to 60 mm. Hg. In many instances the fall was greater. In this dog the pressure remained low, 40 to 50 mm. Hg, until the infusion started, whereupon it rose rapidly. By the end of the infusion the value was about 115 mm. Hg where it remained during the rest of the first day. The heart rate invariably rose after the hemorrhage; in the animal shown in figure 2 it rose from 96 to 156 beats per minute. It continued to increase to 180 until the infusion was given, after the start of which it decreased, leveling off first at 140 and later at 120. On the following day the heart rate was 170, and the blood pressure was 90 mm. Hg. The variations in blood pressure and pulse of all the survivors were of this order, and especially consistent were the second day's findings that the heart rate was 70 to 100 beats per minute above and the blood pressure 20 to 30 mm. Hg below the control value.

Mean values and standard deviations for other circulatory changes and for O_2 consumptions are shown in table 1. The latter when measured about 15 minutes after hemorrhage was found to have decreased an average of 26 per cent. Thereafter, it began to recover in 14 animals but remained the same or fell still lower in 9 dogs. The determinations which were made directly after saline infusion showed an increase

in 13 animals. By calculating the O_2 consumption on the basis of cc/min/kg., the range of variability was reduced from a standard deviation of about 5 per cent to one of about 3 per cent. Little improvement was shown in the metabolic rate according to the final determinations made on the first day, but on the second day the O_2 consumption of all except 5 dogs exceeded the control levels. The control O_2 consumption values for 4 of the 5 dogs were unusually high. Since the heart rates of these animals were also high, there seems little question that the animals were excited during the control period.

The hemorrhage did not greatly alter the arterial O_2 content, but, as expected, the venous O_2 content was greatly reduced. Thus, an average A-V O_2 difference of

TABLE 1. EFFECTS OF HEMORRHAGE AND SALINE INFUSION ON O_2 CONSUMPTION, HEMATOCRIT VALUE, ARTERIAL AND VENOUS O_2 CONTENT, CARDIAC OUTPUT AND PERIPHERAL RESISTANCES

	BEFORE HEMORRHAGE	$\frac{1}{2}$ HR. AFTER HEMORRHAGE	1 HR. AFTER HEMORRHAGE	$\frac{1}{2}$ HR. AFTER INFUSION	$1\frac{1}{2}$ HR. AFTER INFUSION	$10\frac{1}{2}$ HR. AFTER INFUSION
O_2 consumed cc/min/kg.	11.1 \pm 1.5	8.2 \pm 1.4	9.3 \pm 1.2	9.9 \pm 1.4	9.8 \pm 1.0	11.1 \pm 1.6
Hematocrit % R.B.C.	43.2 \pm 4.1	38.9 \pm 3.9	38.9 \pm 3.5	22.5 \pm 2.8	24.3 \pm 3.0	25.2 \pm 2.4
Arterial O_2 vol. %	18.1 \pm 1.6	16.0 \pm 1.7	16.4 \pm 1.5	9.2 \pm 1.0	9.7 \pm 1.1	9.9 \pm 1.1
Mixed venous O_2 vol. %	13.8 \pm 1.8	4.0 \pm 1.8	4.9 \pm 2.2	5.2 \pm 1.4	5.3 \pm 1.1	5.7 \pm 1.3
Cardiac out- put cc/min/kg.	274 \pm 54	74 \pm 22	86 \pm 21	265 \pm 58	229 \pm 44	274 \pm 65
Peripheral re- sistance A.U.	5400 \pm 1500	5900 \pm 1600	4900 \pm 1100	5100 \pm 1300	5400 \pm 1100	3800 \pm 110

The quantities represent mean value with average deviations from the mean and were obtained from measurements on 23 surviving dogs. Time values are also averages.

$$\text{Peripheral resistance} = \frac{\text{mean arterial pressure}}{\text{cardiac output/sec.}} \times 1332 \text{ in } \frac{\text{dynes second}}{\text{cm.}^5} \text{ absolute Units.}$$

A. U. = Absolute Units.

12.0 vols. per cent was observed in contrast to the average control difference of 4.3 vols. per cent. After the infusion the arterial O_2 content was decreased an average of 7.2 vols. per cent by the dilution of the blood. Since changes in arterial O_2 followed closely the changes in the concentration of red cells, it may be inferred that the percentage saturation of the arterial blood remained relatively unchanged. The average venous O_2 content increased only slightly before the start of the infusion, from 4.0 to 4.9 vols. per cent, and continued near this latter value throughout the rest of the day. The cardiac output, reduced to 27 per cent of the control value by the bleeding, showed a slight improvement in 14 of the animals during the hour following hemorrhage. After saline infusion, however, the increase above pre-infusion levels was striking. In 11 dogs the cardiac outputs were higher than the control value, the

average for all 23 dogs being only 4 per cent below the average control value of 274 cc/min/kg. Thus, with the lowered arterial O₂ concentration and with the venous O₂ concentration maintained by the improved outflow of blood from the heart, the A-V difference was decreased to the control range. This favorable condition continued not only for one hour after the infusion but was found to be about the same the following morning, some 19 to 21 hours after blood loss. At this time the average A-V difference was still approximately 4.4 vols. per cent, and the mean value for the cardiac output was the same as the mean control value, 274 cc/min/kg.

Peripheral resistance was calculated according to the method described by Wiggers (22) and the values obtained are included in table 2. There was a wide variation of response as shown by the large standard deviations. In most of the dogs, however, the resistance increased after the bleeding. The average control value for all survivors was 5400 A.U., which increased to a post-hemorrhage value of 5900 A.U. During the hour before the saline there was a decline to an average of 4900 A.U. The measurements following the infusion showed the changes to be highly individual, the mean value being 5100 A.U. immediately afterwards and 5400 A.U. one hour later.

TABLE 2. MEAN VALUES AND AVERAGE DEVIATIONS FROM THE MEAN OF TOTAL AMOUNTS OF CIRCULATING PLASMA PROTEIN

NO. OF SURVIV- ING DOGS	T.P. (a)	T.P. (b)	T.P. (c)	INCREASE IN T.P.	
	gms.	gms.	gms.	gms.	gm./kg.
23	24.8 ± 4.6	11.4 ± 2.6	18.7 ± 3.8	7.2 ± 2.4	0.9 ± 0.2

T.P. (a) = Total plasma protein calculated from plasma volume and plasma protein concentration measured during the control period. T.P. (b) = Total plasma protein lost during the hemorrhage. T.P. (c) = Total plasma protein calculated from measurements made on the following morning.

On the following day every dog, with one exception, showed a decrease in peripheral resistance. The average value at that time was 3800 A.U.

In preliminary experiments (15), 2 dogs, which were given saline equal to 18 per cent of the body weight, developed pulmonary edema. However, in the experiments reported in which saline amounting to 15 per cent of the body weight was administered the only marked edema seen was in the paws and around the eyes. These signs had disappeared by the following morning.

The various determinations made on the 4 non-survivors showed values not strikingly different from the averages for the survivors. No common denominator could be found which would account for all four deaths. Dog 25 had a blood pressure of 100 mm. Hg and a heart rate of 110 beats per minute at the end of the first day. He died about 21 hours after the hemorrhage. Autopsy revealed no cause. Dogs 24 and 26 rallied only temporarily from the post-hemorrhagic hypotension. Their mean pressures rose to 90 and 60 mm. Hg respectively, but half way through the saline injection the pressures began to fall. When put in their cages the pressures were 45 and 18 mm. Hg respectively. No urine could be collected from the bladder; instead profuse diarrhea appeared. The same response was shown by dog 27 except that the mean arterial pressure was better maintained and some urine was formed. In this instance fluid was lost by vomiting as well as by diarrhea.

DISCUSSION

Since 23 of the 27 dogs survived it is of interest to consider how many might have lived if no saline infusion had been given after the hemorrhage. It has been found by Wang *et al.* (23) that 50 per cent survival (L.H.₅₀) can be expected if there is a residual blood volume of 59 cc/kg. one hour after the hemorrhage. In the experiments reported in this paper the blood volume was not measured after the bleeding. Therefore the post-hemorrhagic residual blood volume can only be roughly estimated. With an average control blood volume of 94 cc/kg. and an average hemorrhage volume of 55 cc/kg., an uncompensated residual blood volume of 39 cc/kg. would have been expected. However, during the bleeding, tissue fluid enters the blood vessels, diluting the serum proteins. Walcott (24) found that in 23 dogs the compensatory reserves involved in the early restoration of blood volume ranged from 3 to 18 per cent of the control blood volume, the average being 10.7 per cent. This mean value has been used in Walcott's formula to estimate the residual blood volume at the time of the start of the saline infusion for each dog used in these experiments: $0.107 \text{ B.V.} + (\text{B.V.} - \text{H.V.})$ in which B.V. represents control blood volume and H.V., hemorrhage volume. The average calculated residual blood volume for all of the dogs was 61 cc/kg. for which, according to Wang's figures, a 58 per cent survival might be expected. Even though this estimated residual blood volume is a rough approximation the survival of 23 out of 27 dogs indicates that the infusions were of therapeutic value.

Direct evidence of the beneficial effect of saline on the survival rate of hemorrhage dogs was obtained from experiments on 6 animals (25) which were subjected to a Walcott hemorrhage (14). Except for the fact that no saline infusions were given to these 6 dogs the experimental conditions were the same as for the 27 dogs whose data are being reported in this paper. Only one dog was alive 24 hours after the bleeding. The other 5 died within 2 to 5 hours after the hemorrhage.

Approximately 20 hours after the end of the saline infusion the plasma volume averaged 8 cc/kg. greater than before the hemorrhage. Plasma volume changes before, during and after the infusion can only be inferred from changes in plasma protein concentration and hematocrit values. The similarity in the changes of these two throughout the first day of the experiment show that the plasma compartment increased during the infusion and declined slightly when the inflow was stopped.

On the following day the average plasma protein concentration had increased significantly more than had the average percentage of red cell volume. From the measurements on each dog calculations were made of the total amount of plasma protein in *a*) the control plasma volume, *b*) the plasma lost during hemorrhage, and *c*) the post-infusion plasma volume. Averages of these calculations appear in table 2. When allowance is made for the amount of protein lost during hemorrhage, the total plasma protein found on the second day was in each instance larger than might have been expected. The average increase was 0.9 gm/kg. These results show that the significant increase of the plasma protein concentration on the day after hemorrhage was caused partly by the addition of protein to the circulation rather than entirely by a loss of plasma. The experiments of Beard *et al.* (26) and those of Stewart and Rourke (27) showed similar results when normal salt solution was injected intravenously following a hemorrhage. Ebert *et al.* (28) found that the plasma proteins increased after hemorrhage even though no transfusion was given.

From the average hematocrit values in figure 1 it can be seen that on the day after bleeding the percentage of red cells was approximately equal to the value found after infusion. This does not necessarily mean that the plasma volume remained unchanged, for according to Huber (29) the total red cell volume decreased 24 hours after hemorrhage. Probably a similar change occurred in these experiments.

Objection to the use of saline as a blood substitute has been raised on the grounds that it is too easily lost from the circulatory system, thus yielding only a temporary increase in blood volume. As is well known sodium and chloride ions and water are freely diffusible through capillary walls, and so, limited only by the diffusion gradients of the ions, saline can move quickly into the tissue spaces which are dehydrated by exsanguination. Stewart and Rourke (27), who gave humans large isotonic saline injections over a period of 8 days, found that the salt solution was held in the extracellular fluid compartment. They did not measure how long saline is retained after the end of the infusion.

In the present experiments saline equal to almost twice the control blood volume was infused. The mean arterial pressure never rose above 125 mm. Hg. Much of the injected solution probably entered the interstitial spaces and remained there for at least a few hours. This is confirmed by the observed increase in the weight of the animals and the presence of cutaneous edema. Twenty hours later, however, the extensive diuresis, the weight loss and the disappearance of the edema indicate a general fluid loss, which is probably extracellular. The success with large volumes of isotonic fluid has been explained by Warren, Merrill and Stead (13) who suggest that when enough saline fills the interstitial compartment, the tissue pressure is raised high enough to oppose the capillary hydrostatic pressure. This, they believe, helps to retain saline in the vascular bed so that the blood volume cannot only be restored but also can be maintained at a normal level. In the experiments reported in this paper it appears that though a similar equilibrium between hydrostatic and tissue pressure may have been reached toward the end of the saline infusion, most of the infused solution had been lost by the next day.

The events which occur in the exsanguinated dog result from the actual loss of blood volume. Alterations in the cardiac output (30, 31), O_2 consumption, venous O_2 content and other chemical (32), as well as clinical (18a), observations indicate a reduced blood flow through the tissues, thus bringing about the development of stagnant anoxia (30). The data reported in this paper indicate that isotonic saline administered in sufficiently large amounts restores the total blood flow, for after the infusion the heart, on the average, expelled as much blood as it had before the hemorrhage. To be sure, the O_2 content of arterial and venous blood remained low, but the significant point is that the A-V O_2 difference was restored to the normal value. The venous O_2 content is maintained by the improved blood flow rather than by lack of O_2 utilization. There was close agreement in all arterial O_2 measurements between the hematocrit value and the O_2 content. Thus, at all times the blood received by the tissue cells was of a high percentage saturation. In venous blood, however, the O_2 tension fell to low values after the hemorrhage but was restored by the increased cardiac output to normal limits following the infusion. The next morning this improved cardio-vascular condition was still present. The cardiac output and the

venous O_2 content had increased slightly. Oxygen consumption was restored to the control rate. In spite of the fact that the total blood volume was still 16 per cent below the control measurement, considerable circulatory recovery had taken place.

In the experiments reported here not only was the proportion of surviving dogs high but not one of the dogs which lived developed any of the clinical symptoms of shock, such as apathy, pale, dry mucous membranes of the mouth, cold limbs or collapsed veins (33). The fact that the saline infusion was started as early as one hour after the severe hemorrhage may have prevented the development of the metabolic changes which lead to a condition of shock. By increasing the total systemic blood flow during the hours just after hemorrhage the saline administration may have helped the animal through a critical period until its own defense mechanisms, such as addition of protein to the blood, could be brought into play. On the following day, though little of the infusion fluid remained, the cardiac output was sufficiently restored so that with adequate care and feeding each dog probably could be returned to a healthy, normal condition in time.

SUMMARY

A study has been made of the effects of hemorrhage and of an intravenous infusion of a large volume of isotonic salt solution on the circulatory system. Calculated residual blood volumes average 61 cc/kg. one hour after hemorrhage. Nevertheless, 23 of the 27 dogs survived. In six control experiments 5 dogs died within 2 to 5 hours after the hemorrhage.

The average hematocrit reading was decreased by the saline infusion. Eighteen hours later it had not varied significantly from this post-infusion level. Average values for plasma protein concentration showed a similar decrease following the saline infusion but had increased significantly the following day (fig. 1). Calculation of the total circulating plasma protein showed that after hemorrhage an average of 0.9 gm/kg. of body weight had been added to the blood. After infusion the plasma volume averaged 8 cc/kg. above the control value, but the total blood volume was 15 cc/kg. below the control value. The dogs drank copiously and excreted large volumes of urine.

The average cardiovascular changes can be seen in table 1. Oxygen consumption showed an average reduction of 27 per cent following hemorrhage but after the infusion it was only 10 per cent less than the control values. By the next day the average O_2 consumption was the same as the control rate. The average control cardiac output of 274 ± 54 cc/min/kg. was reduced to one fourth immediately following hemorrhage. The saline inflow brought it up to 265 ± 58 cc/min/kg., and the following day it was 274 ± 65 , the same as the control output. In accord with the hematocrit changes, the average arterial O_2 content fell after the infusion from 16.4 to 9.2 vols. per cent, being slightly increased on the second day. The venous O_2 content, though lowered from 13.7 to 4.0 vols. per cent by the bleeding, rose after the infusion to 5.2 vols. per cent and remained in that range. Thus, the average A-V O_2 difference increased from a control of 4.3 vols. per cent to one of 12.0 vols. per cent after hemorrhage. The infusion restored it to 4.0 vols. per cent and it was not significantly changed 24 hours later. These circulatory changes induced by the large saline infusion offer an explanation of the beneficial effects observed.

The author wishes to express her gratitude to Dr. Magnus I. Gregersen for suggesting this problem and for his advice and encouragement during its course and also to Dr. Walter S. Root for his valuable assistance during the preparation of the manuscript.

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EFFECTS OF CHANGES IN BODY TEMPERATURE AND INSPIRED AIR HUMIDITY ON LUNG EDEMA AND HEMORRHAGE¹

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USING the resistance breathing method described by Zinberg, Nudell, Kubicek and Visscher (1), production of pulmonary edema appeared to be increased when dogs spontaneously developed hyperthermia on warm humid days (2). On cooler days, when hyperthermia was intentionally produced by having the animals breathe 100 per cent humidified air warmed to 37° C., severe pulmonary edema again developed. In order to determine whether the warm humidified air or hyperthermia was the causative factor, it was decided to use guinea pigs where variables could be more easily controlled. Sussman, Hemingway and Visscher (3) have shown that guinea pigs subjected to artificial respiration of 20 mm. Hg positive pressure insufflation by tracheal cannula for 3 to 6 hours regularly develop pulmonary edema. Using shorter time periods and slightly lower pressures to create minimal edema, the animals reported in the paper were studied in regard to environmental, body and airway temperature in addition to the humidity of the air breathed. Hall and Wakefield (4), Jacobsen and Kiyoshi (5) and others mentioned pulmonary edema, hemorrhage and congestion among the pathological findings at autopsy in a small percentage of animals subjected to hyperthermia. Malamud, Haymaker and Custer (6), in a clinico-pathological study of 125 human victims of heat stroke, reported elevated lung weight due to hemorrhage, edema and congestion in all cases. Cardiac hemorrhages were also frequently present, confirming previous findings of Wilson (7).

METHODS

Adult guinea pigs of both sexes weighing between 228 and 729 gm. were employed. They were anesthetized with intraperitoneal sodium pentobarbital, 3.0 mg/100 gm. Artificial respiration by positive pressure tracheal cannula insufflation was maintained throughout. The maximum pressure of insufflation was 18 mm. Hg. Expiration was passive, at atmospheric pressure. Two animals, closely paired for weight, were studied simultaneously on the same respiration pump. One animal acted as a control breathing room air while the second animal, in Series I of the experiment, was required to breathe 100 per cent humidified air warmed to its body temperature from a humidifier interposed in the respirator system. In Series II, the second animal was placed in a warming box to produce hyperthermia while breathing the same room air as its control. In Series III, the second animal breathed warm humidified air in addition to being subjected to the elevated environmental temperature of the warm-

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TABLE I

Series I										Series II										Series III									
CONTROL					HUMIDIFIED AIR AT BODY TEMPERATURE					CONTROL					HYPERTHERMIA					CONTROL					HYPERTHERMIA PLUS HUMIDIFIED AIR AT BODY TEMPERATURE				
$\frac{L}{B} \times 10^4$	$\frac{L}{V}$	Max. body temp. °C.	Notes	$\frac{L}{B} \times 10^4$	$\frac{L}{V}$	Max. body temp. °C.	Notes	$\frac{L}{B} \times 10^4$	$\frac{L}{V}$	Max. body temp. °C.	Notes	$\frac{L}{B} \times 10^4$	$\frac{L}{V}$	Max. body temp. °C.	Notes	$\frac{L}{B} \times 10^4$	$\frac{L}{V}$	Max. body temp. °C.	Notes	$\frac{L}{B} \times 10^4$	$\frac{L}{V}$	Max. body temp. °C.	Notes	$\frac{L}{B} \times 10^4$	$\frac{L}{V}$	Max. body temp. °C.	Notes		
85 ¹	3.26	35.6	S3	116 ²	4.28	38.8	S3	76	2.85	35.8	Si†	93 ²	3.88	43.6	Si†	98	3.20	36.3	Si†	83	3.02	36.3	Si†	83	3.02	42.4	Di†		
86	3.20	36.8	S3	111 ¹	4.01	37.7	S3	77	2.82	35.4	S2	66 ²	2.83	40.2	D2	108	3.81	36.3	Si†	155 ²	6.15	42.8	Di†	155 ²	6.15	42.8	Di†		
103 ¹	6.33	34.8	S3	127 ¹	4.98	34.7	S3	116 ²	4.46	35.2	S2	85 ²	3.48	44.9	D2	67	2.88	37.0	Si†	125 ²	4.83	42.0	Si†	125 ²	4.83	42.0	Si†		
100 ¹	4.10	36.7	S3†	91 ¹	3.35	36.3	S3†	79	3.40	38.2	Si†	184 ²	7.15	44.4	Di†	85 ¹	3.90	37.4	Si†	86 ²	3.59	42.6	Si†	86 ²	3.59	42.6	Si†		
98	3.89	38.0	S3	96	3.89	37.3	S3	78	3.06	37.8	Si†	118 ¹	4.84	43.0	Di†	92	3.99	37.3	Si†	113 ²	4.48	43.8	Di†	113 ²	4.48	43.8	Di†		
84	2.95	35.0	Si†	81	2.94	30.0	Si†	84	3.56	36.8	Si†	123 ²	5.14	44.0	Di†	78	2.98	35.2	Si†	146 ²	4.90	44.8	Di†	146 ²	4.90	44.8	Di†		
76	2.70	37.0	Si†	96	3.68	35.4	Si†	79	3.36	35.3	S2	115 ²	4.29	43.7	D2	84	3.41	36.5	Si†	118 ²	4.54	45.0	Di†	118 ²	4.54	45.0	Di†		
78	2.67	36.0	Si†	80	2.51	34.8	Si†	90 ¹	3.14	36.0	S2	101 ²	3.74	43.8	D2	79	3.61	35.8	S2	119 ²	4.54	45.0	Di†	119 ²	4.54	45.0	Di†		
84	3.08	37.7	Si†	100 ¹	3.55	34.7	Si†	89	3.10	34.4	Si†	89 ¹	3.23	43.4	Di†	106	3.92	36.0	Si†	135 ¹	3.42	43.8	Di†	135 ¹	3.42	43.8	Di†		
76	3.08	35.5	S3	88	3.42	36.4	S3	95 ¹	3.61	33.4	Si†	140 ²	4.69	46.5	Di†	90	3.32	37.7	Si†	107 ²	3.93	44.6	Si†	107 ²	3.93	44.6	Si†		
94	3.53	36.3			3.66	35.6		86	3.28	35.6		109	4.14	44.0		89	3.50	36.6		115	4.32	43.5		115	4.32	43.5			
±26	±1.09			99	±0.70		±11	±11	±0.52			±30	±1.20			±13	±0.40			±27	±0.90			±27	±0.90				

$$\frac{L}{B} = \frac{\text{Lung wt. in grams}}{\text{Body wt. in grams}}$$

$$\frac{L}{V} = \frac{\text{Lung wt. in grams}}{\text{Vent. wt. in grams}}$$
¹ = Occasional hemorrhage, slight congestion. ² = Marked hemorrhage and congestion. S followed by number = Killed at — hours of respiration. D followed by number = Died at — hours of respiration.

ing box. The body and respiratory airway temperatures of both animals, in addition to the room and warming box temperatures, were recorded at 15-minute intervals. The state of the lungs at autopsy was assessed by lung to body weight and lung to ventricle weight ratios as well as by gross examination. Lung weights referred to herein represent combined weight of the lungs.

RESULTS AND DISCUSSION

The lung weight/body weight and lung weight/ventricle weight ratios shown in table 1 reveal that an occasional control animal, subjected to artificial respiration at 18 mm. Hg, develops significant pulmonary edema within 3 hours. Previously published observations from this laboratory (8) show that the normal lung weight/body weight ratio in 26 guinea pigs ranging in weight from 246 to 395 gm. with a mean weight of 310 is 0.0072 immediately after light anesthetization. Thus the three series of 'control' pigs in this study which were treated with positive pressure artificial respiration and had mean ratios of 0.0094, 0.0086 and 0.0089 showed evidence of moderate edema. The same inference can be drawn from the lung weight/ventricle weight data. In the previous study (8) this ratio was found to be 2.41 in the 26 animals killed immediately after anesthetization. The mean 'control' values in the present study all show some elevation above this figure. Thus the stresses of hyperthermia and humidified air are superimposed upon a condition which itself produces slight to moderate edema. The effects of the added stresses are evident in the differences between the control and experimental values in each series. It will be noted that this difference in Series I is 5×10^{-4} for the lung/body weight ratio, and 0.13 for the lung/ventricle weight. Neither of these differences has statistical significance, the respective *P* values according to Fisher's method being 0.45 and 0.75. On the other hand the differences between the control and experimental values in Series II are great, in the case of L/B the difference in the means shows $P = 0.01$ and for L/V, *P* is between 0.02 and 0.05. Likewise in Series III for the difference in means for L/B, $P < 0.01$ and for L/V, $P < 0.02$. Thus in both cases the experimental procedure increased lung weight significantly. There is not a significant difference between the two experimental groups in Series II and III. The morphologic observations upon the lungs showed that edema, congestion and hemorrhage contributed to the lung weight increases. The data at hand do not allow one to determine the precise fractions due to each, but the notations in table 1 indicate that in some cases there was massive edema with only slight congestion or hemorrhage.

It seems to be established that hyperthermia favors the production of the pulmonary lesions observed, and that the humidity of the inspired air is a factor of no great consequence. The mechanism by which hyperthermia may act to produce the above changes has not been elucidated. It may be pointed out that in the experiments on dogs, referred to above (2) measurements of pulmonary vascular pressures (9) showed that the pulmonary venous pressure rose most sharply when the body temperature was elevated. This finding would be consistent with the hypothesis that left ventricular failure was induced by hyperthermia. The pulmonary lesions could be accounted for on this basis.

SUMMARY

Under the conditions studied, hyperthermia favored the production of pulmonary edema, hemorrhage and congestion in guinea pigs. The humidity of the inspired air was a factor of no great consequence in the production of pulmonary lesions in these studies.

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PERSISTENT UNILATERAL RENAL HYPERTENSION IN THE RABBIT¹

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SINCE the work of Goldblatt, the rôle of the kidney in hypertension has been extensively reinvestigated in various laboratory animals. The effect of hypertension per se on the kidney has received scant attention experimentally. If this were to be studied in animals with renal hypertension, it would be necessary to use animals with, what we have called, unilateral renal hypertension—that is hypertension due to the manipulation of one kidney or its blood supply without removing the other kidney. We are presenting the successful production of unilateral renal hypertension in the rabbit.

Most of the work in experimental renal hypertension has been done in the dog, and, unfortunately, in this species the manipulation of both kidneys is almost always necessary in order to get a persistent hypertension (1-5). Persistent unilateral renal hypertension has been produced in the rat (6-9). These experiments have been questioned by some because of the purported occurrence of 'spontaneous' renal disease (10, 11), and even 'spontaneous' hypertension (10) in this animal. Also, the reliability of the methods for determining blood pressure in these experiments is in some doubt (12-14). Unilateral renal hypertension has been reported in the rabbit (15-20), but this work has received scant attention. Possibly the inadequacy of the data pertaining to the persistence of the hypertension, the subjectivity of the blood pressure methods used, or the uncertainty of a truly unilateral origin of the renal hypertension accounts for the lack of recognition of these experiments (2, 3, 8, 9). At any rate, the apparent absence of spontaneous hypertension in the rabbit, suggested the latter as a suitable animal for this study.

METHODS

Production of Renal Hypertension. We have produced persistent renal hypertension by the application of a pre-formed latex capsule to one kidney. Sobin (13) introduced the latex capsule for the purpose of producing hypertension in the rat by bilateral renal application; and Abrams and Sobin (21) did a few bilateral renal encapsulations in the rabbit but did not observe the blood pressure. They did note that the perinephritis due to latex in the rabbit was similar pathologically to the perinephritis due to latex, silk or cellophane in the rat. These foreign substances produce a fibrocollagenous perinephritic hull that apparently compresses the kidney through cicatricial contraction. We chose latex encapsulation instead of silk or cellophane encapsulation or instead of renal artery constriction, only because it appeared to be the technically simplest way to get hypertension in a large percentage of animals.

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Kidney-shaped glass forms were blown from tubing 1 cm. in external diameter. There was a very slight indentation at the 'hilum'. They were made in three sizes to simulate the kidneys of rabbits weighing approximately 4 to 6 pounds. The forms were dipped in liquid latex² two or three times at about one-hour intervals. Sometimes an additional layer of latex was painted on at the 'hilum'. The latex capsule was removed from the glass form by gradually working water under the capsule and then stretching it off. The capsules were then dried and powdered with talc to prevent adherence of opposing surfaces (the capsules were inverted during this procedure). When needed, they were washed with and sterilized in 70 per cent ethyl alcohol and were applied 'wet'.

Under ether anesthesia, the left kidney was exposed by an anterior (trans-peritoneal) or posterior (extra-peritoneal) approach; the adipose capsule and renal fascia were stripped off. The index and middle fingers of both hands supported the medial side of the kidney while the stretched capsule was slipped on with an inverting

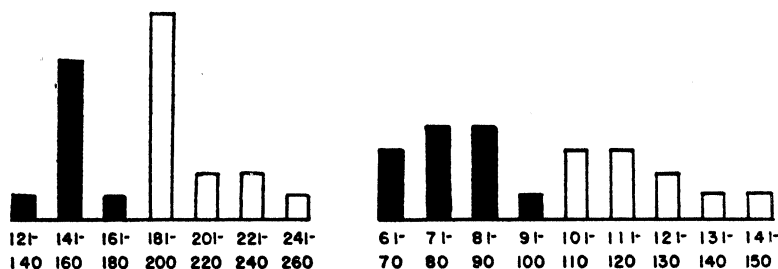


Fig. 1. (left) RELATIVE FREQUENCY (abscissa) of various levels of the highest systolic abdominal blood pressure in mm. Hg (ordinate) of 9 control animals (solid bars) and 15 rabbits that developed hypertension after the latex encapsulation of one kidney (open bars).

Fig. 2. (right) RELATIVE FREQUENCY (abscissa) of various levels of the highest mean ear blood pressure in mm. Hg (ordinate) of 12 control animals (solid bars) and 10 rabbits that developed hypertension after the latex encapsulation of one kidney (open bars).

maneuver by use of the thumbs. The capsules usually fitted a bit loosely, but a few fitted rather snugly. There was no constriction of the hilar structures by the stem or 'hilum' of the latex capsule at the time of application. The right kidney or its blood supply was not removed or manipulated.

Blood Pressure Measurement. Blood pressure was determined by two methods, an indirect and a direct. The former was the abdominal cuff method. Fahr (22) introduced the method, but McGregor (23) has modified and standardized it. The animal was tied down on its back and the cuff was wrapped around the abdomen; the systolic and diastolic blood pressures were determined by auscultation over the aorta under the distal edge of the cuff. In 1120 determinations in 84 normal rabbits, McGregor found the average systolic blood pressure to be 125 mm. Hg, with a range of 90 to 160 mm. Hg. Although this does not represent the true abdominal aorta blood pressure, it bears quite a constant relation to it (24). In 56 determinations in 14 control rabbits we noted a systolic blood pressure over 160 mm. only once, when it was 164 mm. Hg. Because we were desirous of studying animals that were truly hyper-

² The General Latex and Chemical Corporation, Cambridge, Mass.

tensive, rather than merely defining the lower limit of hypertensive blood pressures, we only considered a systolic abdominal blood pressure of 180 mm. Hg or more to be a 'hypertensive' reading.

In most of our animals, the blood pressure was also determined by a second method. The rabbit was placed in a simple warming chamber, and after the central artery of the ear became well dilated, arterial puncture was easily performed with a 22-gauge needle. The mean blood pressure was then observed on a small bore mercury manometer. The method was standardized on 12 normal rabbits; 29 determinations ranged from 60 to 93 mm. Hg. Landis, Montgomery and Sparkman (24)

TABLE I

Rabbit 187: Latex capsule applied on July 9, 1948. Note spontaneous remission in hypertension after Aug. 30, '48.

Date:	7/24/48	8/14	8/19	8/28	8/30	9/23	10/7	10/18	11/9
Abdom. B. P.:	170/120	170/125		195/150		140/98			162/92
Ear B.P.:			105		122		84	79	

Rabbit 153: Latex capsule applied on Mar. 6, 1948. This animal was used for another experiment on May 5, '48.

Date:	3/8/48	3/13	3/20	3/26	4/1	4/3	4/13
Abdom. B.P.:	145/100	142/100	150/100	184/?	176/128	194/?	230/150
Date:	4/14	4/17	4/21	4/24	4/29	5/1	
Abdom. B.P.:	208/154	198/146		216/154		204/168	
Ear B.P.:			112		106		

Rabbit 171: Latex capsule applied May 6, '48. Animal was used for another experiment on July 21, '48. Note fluctuating nature of the hypertension.

Date:	5/11/48	5/22	5/25	6/4	6/7	6/23	6/25	7/13	7/16
Abdom. B.P.:		182/140		145/?		194/142		164/114	
Ear B.P.:	91		84		103		107		107

Rabbit 174: Latex capsule was applied June 8, '48. Animal is still under observation.

Date:	6/18/48	6/29	7/13	7/14	7/29	8/5	8/21	8/26
Abdom. B.P.:	148/114	164/128	204/160		245/196		215/165	205/155
Ear B.P.:			139		124			
Date:	8/30	9/23	10/7	10/18	11/2	11/18	1/26/49	
Abdom. B.P.:	240/160	194/?	204/164		198/158	186/150		
Ear B.P.:			132				109	

using a somewhat similar method, found a systolic blood pressure range of 70 to 98 mm. Hg. Again, we did not consider a mean ear blood pressure as 'hypertensive' unless it was at least 100 mm. Hg. Furthermore, when we speak of a 'hypertensive' animal, we are referring only to those that have had at least two hypertensive readings on different days by one or both of the above blood pressure methods.

RESULTS AND DISCUSSIONS

Twenty-two rabbits thus far have survived the latex encapsulation procedure. Fifteen of them or just over two thirds, have developed hypertension (figs. 1 and 2). The highest mean ear blood pressure we recorded was 148 mm. Hg, and the highest systolic abdominal blood pressure was 245 mm. Hg. The first hypertensive reading usually was noted between the 15th and 66th day after renal encapsulation, but in

one rabbit the first hypertensive reading did not occur until the 120th day after surgery, and in another rabbit it occurred as early as the 5th post-operative day (blood pressures were not taken during the first few post-operative days). In the latter rabbit, one would surmise that the latex capsule itself or its contained exudate, rather than a contracting perinephritic hull, compressed the kidney and so initiated the hypertension.

Nine of the 15 hypertensive rabbits were used for another experiment 18 to 111 days after their first hypertensive reading. Of the remaining 6, one died 47 days after the first hypertensive reading, 2 had a spontaneous and apparently permanent remission 11 to 34 days after the first hypertensive reading, and the other 3 are still exhibiting hypertension 115, 116 and 125 days after the first hypertensive reading. This represents a considerable period of time in the life of a rabbit, so we feel justified in referring to this as persistent unilateral renal hypertension. In table 1 we have included the protocols of 4 of the hypertensive rabbits, as representative of that group.

CONCLUSIONS AND SUMMARY

Persistent unilateral renal hypertension has been produced in the rabbit. A latex capsule was put on one kidney and the other kidney or its blood supply was not manipulated. This preparation may have usefulness in the study of the effects of hypertension per se on the normal kidney. One could also study the course of hypertension after the removal of the source in such a preparation. A simple method for obtaining repeated direct mean blood pressures in the unanesthetized rabbit has also been presented.

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EFFECTS OF REMOVAL OF THE 'ISCHEMIC' KIDNEY IN RABBITS WITH UNILATERAL RENAL HYPERTENSION, AS COMPARED TO UNILATERAL NEPHRECTOMY IN NORMAL RABBITS¹

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IN A previous paper (1) we presented a method for the production of *unilateral* renal hypertension, that is, hypertension due to unilateral renal or reno-vascular manipulation with the other kidney intact. It consisted of the latex encapsulation of one kidney. Blood pressure was determined by two methods, an indirect and a direct. The former was the abdominal cuff method, as modified by McGregor (2); he found the average systolic blood pressure to be 125 mm. Hg, with a range of 90 to 160 mm. Hg. In the second method, the rabbit was placed in a simple warming chamber, and after the central artery of the ear became well dilated, arterial puncture was easily performed with a 22-gauge needle. The mean blood pressure was then observed on a small bore mercury manometer. We found the normal range of blood pressure to be 60 to 93 mm. Hg. Hypertension, that is, two or more hypertensive readings, by either one or both of the blood pressure methods used, was produced in 15 of 22 rabbits. A 'hypertensive' reading was defined as a systolic blood pressure of at least 180 mm. Hg by the abdominal cuff method, or a mean blood pressure of at least 100 mm. Hg by our ear method in the central artery of the ear. It was noted that this hypertension may persist for a considerable period of time. Three rabbits still exhibited hypertension 115, 116, and 125 days after their first hypertensive reading; most of the others were used for the experiment to be presented shortly.

In the work presented in this paper we were interested in investigating whether the continued high blood pressure sets up mechanisms that tend to perpetuate the hypertension. In other words, is a vicious circle brought about? This can be readily studied in the rabbit with unilateral renal hypertension since the original cause can be removed at will and the animal studied thereafter.

PROCEDURE

Our general plan of attack was to produce hypertension and then, after varying intervals, to remove the 'ischemic' kidney and follow the blood pressure thereafter. To serve as controls for this work, and because one investigator has reported that unilateral nephrectomy causes a rise in blood pressure in rabbits (3), we also subjected normal rabbits to unilateral nephrectomy and followed the blood pressure thereafter.

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Effect of Unilateral Nephrectomy on the Blood Pressure of Normal Rabbits. Nine normal rabbits were subjected to unilateral nephrectomy. Blood pressures were followed by the two blood pressure methods used in our previous paper; 33 of 34 determinations by the ear method, over a period up to 209 days after surgery, were normal. That is, they ranged from 62 to 90 mm. Hg, as compared to a range of 58 to 93 mm. Hg in 32 observations in our 12 normal controls (fig. 1). However, one ear blood pressure determination, in one of the unilaterally nephrectomized rabbits, was 109 mm. Hg, a hypertensive reading by our definition. Since the three previous and two subsequent ear blood pressure determinations in this rabbit were non-hypertensive, we do not classify this animal as one with hypertension. Further, none of these 9 unilaterally nephrectomized rabbits exhibited any hypertensive readings in 51 determinations by the abdominal cuff method; the range of systolic blood pressure was 120 to 164 mm. Hg.

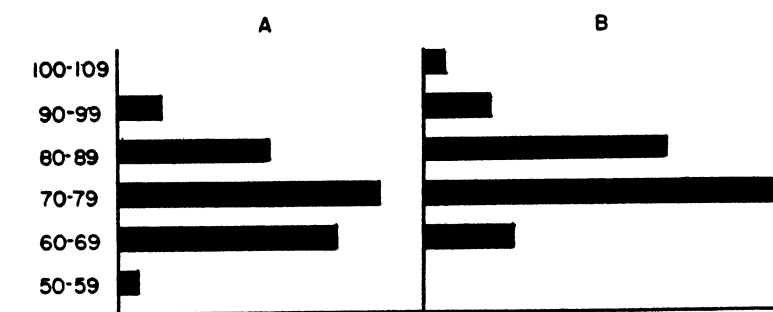


Fig. 1. RELATIVE FREQUENCY (abscissa) of various levels of mean ear blood pressure in mm. Hg (ordinate). A) 32 determinations in 12 normal rabbits; B) 34 determinations in 9 normal rabbits after unilateral nephrectomy.

Effect of Removal of the 'Ischemic' Kidney on the Blood Pressure of Rabbits with Unilateral Renal Hypertension. Seven rabbits with hypertension were subjected to removal of the encapsulated kidney 18 to 66 days after their first hypertensive reading. The hypertension was still present after that operation in 4 of the animals. It finally disappeared spontaneously 23, 48, and 152 days after the operation in 3 of these 4 rabbits, and one rabbit was used for another experiment 11 days after the operation. The blood pressure fluctuated considerably from hypertensive to non-hypertensive levels before it reverted to non-hypertensive or completely normal levels; this was especially true in the rabbit that had a persistence of hypertension for 152 days. The other 3 hypertensive rabbits, that were subjected to removal of the encapsulated kidney, did not exhibit any further hypertensive readings (the first reading was not taken until the third to fifth post-operative day). Even the latter three, however, exhibited blood pressures above the usual upper limits of normal for at least the first few post-operative weeks, i.e., abdominal systolic blood pressures of 160 to 180 mm. Hg.

There appeared to be a positive correlation between the height of the blood pressure and duration of hypertension after removal of the encapsulated kidney. Two of the 4 rabbits that exhibited this persistence of hypertension had one or two

mean ear blood pressures of at least 110 mm. Hg. Further, the latter 2 and another one of these 4 rabbits exhibited two to four systolic abdominal blood pressures of at least 200 mm. Hg. The blood pressures of the 3 rabbits that did not show a persistence of hypertension after the operation were all under the above values.

We have also checked these results in seven rabbits made hypertensive by partially constricting one renal artery with the other kidney intact (4, 5). The manipulated kidney was removed 3 to 80 days after the first hypertensive reading. The hypertension was still present after surgery in 3 of these 7 rabbits. These 3 animals remained hypertensive for at least 72, 103, and 103 days, when they were used for another experiment. In this group of 7 rabbits, one could not draw any conclusions about the relation of the level of the blood pressure to the persistence of hypertension after removal of the 'ischemic' kidney. Possibly, this is related to the

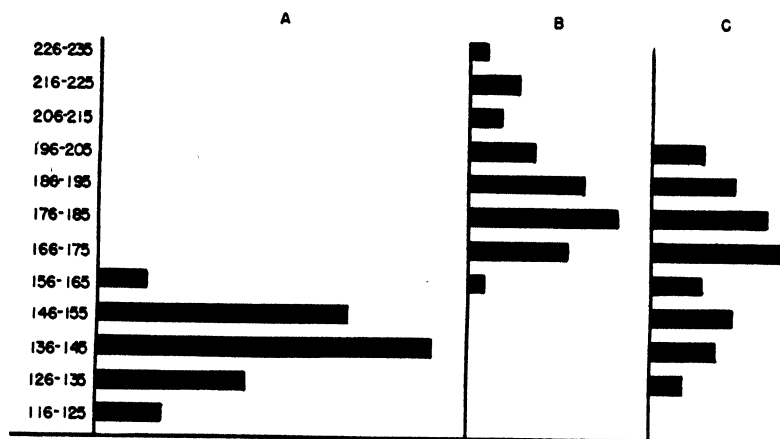


Fig. 2. RELATIVE FREQUENCY (abscissa) of various levels of systolic abdominal blood pressure in mm. Hg (ordinate). A) 51 determinations in 9 normal rabbits after unilateral nephrectomy; B) 33 determinations in 7 hypertensive rabbits; C) 37 determinations in the same 7 rabbits after removal of the 'ischemic' kidney (these animals remained hypertensive for various periods of time).

milder and more equal degree of hypertension that existed in this group. In considering the presence of any correlation between the duration of hypertension before removal of the 'ischemic' kidney and the persistence of hypertension after that operation, we can combine the findings in the two groups of rabbits. In 14 rabbits subjected to removal of the 'ischemic' kidney, in whom the duration of hypertension ranged from 3 to 80 days, there did not appear to be a notable positive correlation between the duration of hypertension before and the persistence of hypertension in 7 of the animals after that operation, except at the extremes in duration. In figure 2 and table 1 we have presented these data. The duration of hypertension is probably somewhat greater than as presented, because of the somewhat lengthy interval between blood pressure readings at times.

DISCUSSION

Similar results have been reported in the rabbit (3), and in the rat (6-9). In the dog, however, removal of the 'ischemic' kidney brings the blood pressure down

to normal within 24 hours (10-12). In man, the 'cure' of hypertension of supposed unilateral renal origin by the removal of the diseased kidney is still a controversial matter.

Friedman, Jarman, and Klemperer (7) were apparently the first investigators to show a persistence of hypertension after removal of the ischemic kidney in animals—the rat—with unilateral renal hypertension. Wilson and Byrom (6) have noted that removal of the ischemic kidney, in rats with unilateral renal hypertension, may revert the blood pressure to normal, even in those rats that have developed the malignant syndrome. Grollman (3), in 5 rabbits with unilateral renal hypertension, removed the manipulated kidney about 10 to 14 weeks after it was made ischemic by distortion with cotton tape; he noted a persistence of hypertension in all. He does not mention how long the hypertension had existed or how long these animals

TABLE 1

RABBIT NO.	PREOPERATIVE DURATION OF HYPERTENSION	POST-OPERATIVE PERSISTENCE OF HYPERTENSION	RABBIT NO.	PREOPERATIVE DURATION OF HYPERTENSION	POST-OPERATIVE PERSISTENCE OF HYPERTENSION
	days	days		days	days
70	3	<22	175	33	<5
71	3	<17	153	35	48
60	4 ¹	<9	170	37	11 ³
133	11	<4 ⁴	147	52	<5
45	17 ¹	72 ³	148	66	<3
176	18	23	89	76	103 ³
150	32	152 ²	92	80	103 ³

¹ True duration of hypertension is probably somewhat greater than this, because of the long interval between blood pressure readings in these animals.

² The blood pressure was very fluctuant with frequent normal readings.

³ *Rabbit 170* was used for another experiment at that time. *Rabbits 89* and *92* were probably still hypertensive when used for another experiment 10 days later. *Rabbit 45* was not followed thereafter.

⁴ Not followed adequately.

were followed after removal of the 'ischemic' kidney. By the same method of determining blood pressure, he also observed hypertension after unilateral nephrectomy in normal rabbits, a result in disagreement with the work of most investigators, and with our results. Pickering (13) found no elevation of blood pressure after removal of one or both kidneys in the rabbit. Braun-Menendez *et al.* (11) state that it has been shown that unilateral nephrectomy is not followed by any appreciable change in blood pressure in any tested animal. Pickering (13) has noted that 'bilateral' renal hypertension of at least 7 weeks' duration, in the rabbit, persists even after both manipulated kidneys are out (in the several days the animals survive). In rabbits, with 'bilateral' renal hypertension of up to 8 days' duration, there was no persistence of hypertension; it disappeared in a few hours after the second kidney was removed, just as he noted to be true after cessation of a prolonged renin injection.

Our results support the view (3, 6-9, 13, 15) that there is a 'change of mechanism' in the course of renal hypertension. Possibly a continued hypertension can

produce effects which will maintain the hypertension after the original cause is removed. A high blood pressure that has lasted for some time does produce changes in the body, although undoubtedly most of these effects are unknown to us. However, in the category of anatomical changes, we might cite the hypertrophy of the left ventricle. In the category of physiological changes we may cite the normal pulse rate in the presence of a raised blood pressure. If we raise the blood pressure of a normal rabbit (by compression of the abdominal aorta) to a hypertensive level, the heart rate becomes slowed. The heart rate of the hypertensive rabbit will respond to a rise of blood pressure above its control level, i.e. a slowing following compression of the abdominal aorta. It would seem that this reflex mechanism might be dulled to the ordinary blood pressure stimulus and needed a more pronounced stimulus, or we may look on it as a change in the 'set' of a regulatory mechanism.

These known effects of a continued high blood pressure probably play a rôle in the persistence of hypertension after removal of the ischemic kidney. Undoubtedly other factors are involved. This self-perpetuation of some disturbance in function after removal of the inciting cause is not unknown in physiology. There is much evidence to support the concept that long continued induced hyperglycemia will result in a hyperglycemia that persists after the animal is put back on a normal regime. It is believed that the high sugar level over-strains the islet tissue of the pancreas so that thereafter it functions inadequately. Adiposity tends to perpetuate itself by limiting the activity of the individual. A weakened heart action is liable to produce a vicious circle since it leads to reduced coronary blood flow. In these examples the mechanisms bringing about the self-perpetuation are quite different and may only be indirectly related to the characteristic itself, as in the case of adiposity.

The non-operated kidney may play an important rôle in this self-perpetuation. It may be affected by the high blood pressure per se so that its functioning is so altered that it aggravates the pressure raising process by secreting a pressor substance or by some other means. On the other hand, the changes produced on the experimental kidney may in some specific manner result in an alteration of the other kidney by some mechanism analogous to sympathetic ophthalmia. Only in this latter case would our experimental method lose some of its value as a research tool in the study of hypertension.

CONCLUSIONS AND SUMMARY

None of the 8 normal rabbits subjected to unilateral nephrectomy developed hypertension. Seven of the 14 rabbits with unilateral renal hypertension showed a persistence of hypertension for some time after removal of the 'ischemic' kidney.

We believe that a long continued high blood pressure is a characteristic which tends to be self-perpetuating. This might be due to overstraining or 'dulling' of a regulatory mechanism, or might be due to indirect effects, such as renal damage or widespread vascular damage.

Since our work, and more especially work in the rat, shows that removing the ischemic kidney in unilateral renal hypertension may not 'cure' the hypertension perhaps it is not safe to assume that man will be cured of supposed unilateral renal

hypertension by the removal of the involved kidney. This operation was founded in part on the observation that in the dog, removal of the ischemic kidney, during the course of the temporary unilateral renal hypertension that can be produced in that animal, promptly brought the blood pressure down to normal.

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INFLUENCE OF SODIUM LOAD ON SODIUM EXCRETION¹

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THE relation of sodium to heart failure and arterial hypertension has emphasized the need for more complete knowledge of the factors which regulate its excretion. Existing data relative to overall sodium metabolism have been summarized in recent reviews and monographs (1). The purpose of the present study was to measure the alterations in renal function which follow the administration of sodium chloride and to determine the mechanism through which increased sodium intake alters sodium output.

METHODS AND MATERIALS

Renal sodium excretion is presumably determined by the relation between the amount of sodium filtered by the glomeruli and the fraction of filtered sodium which escapes tubular reabsorption. On the assumption that glomerular urine is an ultrafiltrate of plasma, the magnitude of sodium filtration was estimated in these studies by the expression:

$$GFR_{Na} = GFR \times B_{Na}$$

Where: GFR_{Na} = Sodium filtration rate (mEq/min.);

GFR = Glomerular filtration rate (cc/min.) as measured by the mannitol technique (2);

B_{Na} = Plasma sodium concentration (mEq/cc.) as measured by the uranium acetate method (3).

The influence of tubular activity on sodium excretion was estimated by the expression:

$$TRF_{Na} = \frac{U_{Na} \cdot V}{GFR_{Na}} \times 100$$

Where: TRF_{Na} = Sodium tubular rejection fraction (mEq. sodium excreted per 100 mEq. filtered).

U_{Na} = Urinary sodium concentration (mEq/cc.).

V = Urine flow (cc/min.).

GFR_{Na} = Sodium filtration rate (mEq/min.).

Renal plasma flow was measured by the para-aminohippurate technique (4).

The relative importance of the glomerular and tubular mechanisms in compensating for changes in sodium intake was studied in a series of 12 normal dogs. The weights of the animals varied between 8 and 26 kg. Surface area was calculated by the formula: $Wt_{kg}^{2/3} \times 0.107 = m^2$.

On the preceding day 50 cc. of water per kilogram were given by stomach tube. This dose was repeated about 15 hours before the experiment. Anesthesia was accomplished with dial urethane², 0.7 cc/kg. given intraperitoneally. Blood pressure was recorded continuously from the left common carotid artery through a cannula attached to a mercury manometer. Electrocardiograms were recorded from subcutaneous needles inserted in the right upper and left lower extremities. All blood

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² A generous supply of dial urethane was made available through the courtesy of Ciba Pharmaceutical Products, Inc., Summit, N. J.

samples were obtained from the femoral artery through an indwelling needle. Infusions were made into the left jugular vein by a constant speed infusion pump. Urine was collected through an indwelling soft rubber catheter. The bladder was washed out with distilled water after each collection and emptied completely by blowing out with air.

Basal renal functional values were determined for each animal during two or more 30-minute control clearance periods. Sodium loads were then imposed by the intravenous administration of test solutions of sodium chloride which ranged in concentration from 0.86 to 30 per cent. The test solutions were infused at constant speeds for periods of 1 to 10 hours. Rates of administration and the values which were measured and derived are indicated in table 1. All chemical analyses were done in duplicate. Pre-injection samples were used for blank determinations.

RESULTS

Survival. Animals infused at rates in excess of 13 milliequivalents of sodium per minute per square meter died at the end of 30 to 90 minutes. Death was preceded by sudden decrease in heart rate, disappearance of the P wave, increased amplitude of the T wave, and development of ventricular premature contractions. This phase was followed by ventricular asystole or fibrillation with rapidly fatal termination. Animals injected at rates less than 8.5 mEq. Na/min/m² outlived periods of observation from 3½ to 10 hours in duration.

Sodium Excretion and Tubular Rejection Fraction. The time until maximum output of sodium varied from 30 minutes to 8 hours after the start of infusion, and was related in general to the rate of sodium administration (table 1 and fig. 1). The maximum excretory rates were 40 to 500 times higher than the basal levels, depending upon the conditions of the experiment as discussed below.

In each animal the changes in sodium excretion were paralleled by corresponding alterations in the sodium tubular rejection fraction (fig. 2). This relationship appeared to be independent both of the concentration of sodium chloride solution infused and of the plasma sodium level. The maximum tubular rejection fraction observed represented approximately 42 per cent of the sodium filtered.

Glomerular Filtration Rate. Infusion of sodium chloride solutions did not produce consistent changes in glomerular filtration rate. In 5 animals infusion of the test solution was followed by a fall in filtration although sodium excretion increased. In those cases in which the filtration rate rose its highest level never exceeded twice the control value. In no instance did the greatest output of sodium occur during the period of maximum glomerular filtration.

Renal Plasma Flow and Filtration Fraction. Administration of salt solutions consistently resulted in a rise in renal plasma flow, which in some instances attained a value two and one-half times the control figure. Due to the magnitude of this change, the filtration fraction fell below the basal value in 5 experiments.

The rise in plasma flow usually occurred early and preceded the highest level of sodium output. As a consequence, a sustained or increasing rate of sodium excretion was often manifested in the face of a falling renal plasma flow (table 1, animals 1, 2, 5, 6, 7).

Water Excretion. The volume of urine flow increased concurrently with the rise in sodium excretion. The increase was due almost entirely to diminished tubular reabsorption of water, rather than to a change in glomerular filtration rate.

TABLE I

I	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
PERIOD	INJECTION RATE		URINE	GFR	H ₂ O BAL- ANCE	RFF	FF	U'Na	U'Na-V	B'Na	GFR'Na	Na BAL- ANCE		EXTRA-CELL- ULAR Na	Na SPACE	Na SPACE	CELL. H ₂ O CHANGE	HEMATOCRIT	MEAN BP	
	Na	H ₂ O										mEq/ min/ m ²	cc/ min/ m ²							mEq/ min/ m ²
		mEq/ min/ m ²	cc/ min/ m ²	cc/ min/ m ²	cc/ min/ m ²	cc/ min/ m ²	cc/ min/ m ²	%	mEq/ cc.	mEq/ min/ m ²	mEq/ cc.	mEq/ min/ m ²	mEq/ min/ m ²	mEq/ min/ m ²	mEq/ m ²	cc/m ²	cc/m ²	cc/m ²	Vol. %	mm. Hg
Dog 1, female; 0.445 sq. m. surface area																				
Control Average (2 x 30')			0.66	49	1.35	131	37	0.038	0.020	0.155	7.65	0.27		540	3453					
T ₁ (30')	3.00	2.20	0.58	42	1.40	27	155	0.106	0.094	0.137	5.74	1.64		653	4312	859	-832		138	
T ₂ (60')	3.00	2.29	3.28	37	8.78	-59	130	0.171	0.502	0.174	6.48	8.07		310	4630	1177	-1236		115	
T ₃ (60')	3.00	2.20	4.16	48	8.77	-203	148	0.173	0.720	0.191	9.13	7.09		1035	51308	1678	-1881		116	
T ₄ (60')	3.00	2.29	2.59	34	7.62	-247	94	0.287	0.743	0.211	7.13	10.40		1219	5520	2067	-2314		106	
Dog 2, male; 0.450 sq. m. surface area																				
Control Average (2 x 30')			1.15	120	0.96	355	34	0.086	0.098	0.152	18.3	0.54								
T ₁ (30')	3.80	2.27	1.71	103	1.65	-9	405	0.220	0.376	0.141	14.5	2.58							130	
T ₂ (60')	3.80	2.27	4.40	106	4.24	-171	364	0.299	1.34	0.178	18.8	7.12							122	
T ₃ (60')	3.80	2.27	4.20	119	3.60	-315	280	0.207	1.27	0.186	22.0	5.77							117	
T ₄ (60')	3.80	2.27	4.51	76	5.90	-487	242	0.317	1.43	0.207	15.8	9.02							113	
Dog 3, male; 0.658 sq. m. surface area																				
Control Average (2 x 30')			0.52	72	0.72	208	34	0.067	0.036	0.152	11.0	0.32		822	5400					
T ₁ (30')	35.7	6.94	10.03	82	12.30	-112	255	0.230	2.30	0.189	15.5	14.85		1841	8271	2871	-2083		143	
T ₂ (20')	35.7	6.94	26.90	71	31.90	-427	259	0.300	6.79	0.227	16.3	41.80		2543	9587	4187	-4614		154	
Dog 4, male; 0.652 sq. m. surface area																				
Control Average (2 x 30')			0.76	62	0.88	190	33	0.060	0.047	0.170	10.5	0.45		1024	6010					
T ₁ (26')	72.2	14.05	8.59	55	15.54	81	226	0.246	2.11	0.261	14.4	14.60		2620	6771	761	-680		108	

Dog 5, female; 0.534 sq. m. surface area

Control Average (2 x 30')	17.1	3.74	0.54	91	0.61	227	37	0.013	0.0032	0.154	14.0	0.04	1050	6830	—162	87	145
T ₁ (20')	17.1	3.74	5.90	82	7.16	-75	33	0.103	1.14	0.189	15.6	7.32	1378	6658	—162	87	183
T ₂ (12')	17.1	3.74	13.27	75	17.61	-216	41	0.204	2.71	0.219	16.9	16.45	1534	6836	16	-232	115

Dog 6, female; 0.417 sq. m. surface area

Control Average (2 x 30')	14.5	8.48	0.52	84	0.61	230	39	0.016	0.009	0.111	11.8	0.08	607	4310	1000	—935	123
T ₁ (20')	14.5	8.48	3.28	77	4.28	65	29	0.184	0.603	0.148	11.4	5.31	866	5310	1053	—935	101
T ₂ (20')	14.5	8.48	12.60	77	16.40	-35	312	0.194	2.46	0.196	15.0	16.30	1119	5363	1053	—1088	94
T ₃ (20')	14.5	8.48	15.00	68	21.93	-201	300	0.234	3.51	0.226	15.4	22.75	1321	5627	1317	—1518	96
T ₄ (20')	14.5	8.48	15.83	58	27.50	-371	276	0.254	4.01	0.250	14.4	27.95	1533	5688	1378	—1749	103
T ₅ (7')	14.5	8.48	7.20	27	26.55	-390		0.309	2.22	0.278	7.52	20.50	1005	5601	1351	—1741	71

Dog 7, male; 0.681 sq. m. surface area

Control Average (2 x 30')	8.30	4.84	0.49	74	0.67	301	25	0.043	0.213	0.157	11.7	0.14	778	4957	598	—544	52
T ₁ (20')	8.30	4.84	1.29	79	1.64	54	302	0.227	0.281	0.159	12.5	1.34	937	5555	598	—544	46
T ₂ (20')	8.30	4.84	5.33	83	6.46	11	335	0.232	1.24	0.177	14.0	8.48	1047	5735	778	—767	43
T ₃ (20')	8.30	4.84	11.73	87	13.49	-138	433	0.244	2.87	0.187	16.3	17.62	1165	6037	1080	—1218	39
T ₄ (20')	8.30	4.84	21.44	95	22.50	-479	525	0.246	0.926	0.108	18.9	20.50	1255	6014	1057	—1536	44
T ₅ (20')	8.30	4.84	22.50	78	28.81	-828	378	0.261	1.53	0.216	16.9	34.90	1320	5863	906	—1734	50
T ₆ (20')	8.30	4.84	16.15	70	23.16	-1057	332	0.292	4.72	0.233	16.2	20.05	1410	6049	1092	—2140	52
T ₇ (20')	8.30	4.84	14.83	70	21.13	-1270	300	0.300	4.30	0.234	16.4	26.15	1495	6231	1274	—2544	52
T ₈ (20')	8.30	4.84	11.74	71	16.60	-1488	257	0.335	3.94	0.247	17.5	22.55	1476	5772	815	—2303	94
T ₉ (20')	8.30	4.84	11.31	59	19.11	-1622	201	0.314	3.55	0.264	15.7	22.65	1588	5854	807	—2510	55
T ₁₀ (20')	8.30	4.84	6.02	25	23.70	-1652	100	0.332	2.00	0.277	7.04	28.42	1711	6053	1096	—2748	58

Dog 8, female; 0.410 sq. m. surface area

Control Average (2 x 30')	0.596	4.06	0.97	69	1.41	149	43	0.003	0.003	0.156	10.8	0.02	690	4423	84	89	56
T ₁ (60')	0.596	4.06	0.68	71	0.97	173	41	0.009	0.006	0.135	11.0	0.06	721	4507	84	89	55
T ₂ (60')	0.596	4.06	0.51	62	0.83	349	44	0.008	0.004	0.161	10.1	0.04	60	751	4628	205	144
T ₃ (60')	0.596	4.06	0.73	69	1.07	557	132	0.011	0.008	0.166	10.9	0.07	91	781	5004	581	24
T ₄ (60')	0.596	4.06	1.34	78	1.73	649	140	0.030	0.041	0.152	11.8	0.33	118	829	984	335	50
T ₅ (60')	0.596	4.06	1.83	79	2.31	756	182	0.057	0.105	0.146	11.4	0.92	145	835	5716	1293	537
T ₆ (60')	0.596	4.06	2.37	56	4.22	827	218	0.072	0.220	0.146	8.26	2.07	166	856	5625	1202	375
T ₇ (60')	0.596	4.06	1.51	98	1.54	944	181	0.105	0.107	0.160	15.7	1.01	186	876	5630	1207	263
T ₈ (60')	0.596	4.06	1.51	117	1.29	1075	203	0.067	0.101	0.151	17.7	0.57	213	903	5986	1563	488
T ₉ (60')	0.596	4.06	1.29	140	0.92	1201	249	0.047	0.060	0.151	21.1	0.28	238	928	6187	1764	563
T ₁₀ (60')	0.596	4.06	1.10	123	0.89	1328	195	0.080	0.088	0.149	18.3	0.48	260	950	6390	1967	639

TABLE I—Continued

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
PERIOD	INJECTION RATE		URINE	GFR	H ₂ O BAL- ANCE	H ₂ O BAL- ANCE	RPF	FF	U _{Na}	U _{Na} ·V	B _{Na}	GFR _{Na}	TRF _{Na}	Na BAL- ANCE	EXTRA-CELL. IAR Na	Na SPACE	Na SPACE	CELL. H ₂ O CHANGE	HEMATOCRIT	MEAN BP
	Na	H ₂ O																		
	mEq./ min/ m ²	cc./ min/ m ²	cc./ min/ m ²	cc./ min/ m ²	cc. cc.	cc./ min/ m ²	cc./ min/ m ²	%	mEq./ cc.	mEq./ min/ m ²	mEq./ cc.	mEq./ cc.	mEq./ min/ m ²	mEq./ cc./ 100 mEq./ filtered	mEq./ m ²	mEq./ m ²	cc./m ²	cc./m ²	cc./m ²	Vol. %
Dog 9, male; 0.434 sq. m. surface area																				
Control Average (2 x 30')																				
T ₁ (60')	2.02	19.83	0.91	71	1.26	1148	215	38	0.013	0.012	0.146	10.4	0.12	178	661	4527	1103		60	101
T ₂ (60')	2.92	19.83	1.15	70	1.67	2113	254	27	0.0778	0.090	0.146	10.2	0.88	178	840	5630		45	55	108
T ₃ (60')	2.92	19.83	2.77	69	4.04	2113	222	31	0.144	0.401	0.152	10.4	3.84	343	1001	6657	2130	17		120
T ₄ (60')	2.92	19.83	4.69	68	6.83	3065	226	30	0.136	0.635	0.149	10.3	6.21	524	1186	7859	3332	-267		116
T ₅ (60')	2.92	19.83	5.53	72	7.75	3915	233	31	0.116	0.643	0.152	10.9	5.91	697	1359	8858	4301	-386	38	131
T ₆ (60')	2.92	19.83	3.76	67	5.51	4915	198	34	0.124	0.465	0.155	10.4	4.46	875	1537	10487	5960	-1045	37	143
T ₇ (60')	2.92	19.83	4.45	83	5.35	5865	203	41	0.133	0.591	0.139	11.6	5.13	1054	1716	11744	7217	-1352	44	120
T ₈ (60')	2.92	19.83	4.24	74	5.74	6595	165	45	0.122	0.517	0.152	11.3	4.60	1200	1861	12854	8327	-1732	37	123
T ₉ (60')	2.92	19.83	4.38	69	6.36	7375	184	37	0.121	0.533	0.139	9.00	5.92	1352	2013	14516	9989	-2614	36	132
Dog 10, female; 0.474 sq. m. surface area																				
Control Average (2 x 30')																				
T ₁ (60')	2.67	18.1	0.41	89	0.46	901	171	52	0.040	0.017	0.138	12.2	0.14	139	807	5833			49	125
T ₂ (60')	2.67	18.1	3.18	90	3.23	1511	233	42	0.120	0.383	0.137	13.5	2.84	350	945	6925	1992	-191	41	114
T ₃ (60')	2.67	18.1	8.80	90	8.90	1511	376	26	0.117	1.03	0.137	13.5	7.60	350	1056	7738	1995	-394	36	118
T ₄ (60')	2.67	18.1	12.69	96	13.20	1667	403	24	0.139	1.76	0.137	13.1	13.79	279	1086	7953	2120	-453	30	116
T ₅ (60')	2.67	18.1	12.25	93	13.21	2044	308	30	0.135	1.66	0.137	12.7	13.10	345	1151	8433	2600	-556	30	136
T ₆ (60')	2.67	18.1	11.45	96	11.79	2534	358	27	0.127	1.45	0.137	13.1	11.10	417	1223	8959	3126	-592	31	124
T ₇ (60')	2.67	18.1	10.75	139	7.74	2884	300	49	0.133	1.44	0.137	19.0	7.56	491	1298	9597	3674	-700	35	133
T ₈ (60')	2.67	18.1	8.85	102	8.55	3364	314	33	0.140	1.22	0.137	14.0	8.73	568	1374	10664	4231	-867	35	132
T ₉ (60')	2.67	18.1	7.23	121	5.98	3994	263	46	0.113	0.821	0.137	16.5	4.97	672	1478	10328	4995	-1001	30	146

Dog 11, male; 1.06 sq. m. surface area

Control Average (2 x 30')	5.55	5.9	1.06	144	40	0.022	0.012	0.158	9.65	0.14	1409	8900		65				
T ₁ (60')	8.50	1.11	5.9	1.90	403	161	37	0.021	0.023	0.158	9.30	0.25	68	1476	9328	428	-25	156
T ₂ (60')	8.50	2.74	97	2.82	711	169	58	0.032	0.086	0.158	15.3	0.56	138	1547	9883	933	-272	158
T ₃ (60')	8.50	3.63	82	4.40	1058	172	48	0.057	0.209	0.155	11.7	1.60	202	1610	10173	1273	-205	158
T ₄ (60')	8.50	3.52	68	5.18	1273	182	37	0.090	0.348	0.162	12.0	3.20	250	1659	10199	1209	-26	158
T ₅ (60')	8.50	3.76	84	4.35	1542	193	44	0.126	0.472	0.166	14.0	3.40	295	1703	10175	1275	267	155
T ₆ (60')	8.50	5.24	57	3.92	1930	167	45	0.157	0.352	0.169	9.70	3.70	350	1759	10535	1635	295	156
T ₇ (60')	8.50	2.69	67	4.07	2267	173	41	0.157	0.422	0.166	11.0	3.80	308	1807	11171	2271	-4	161
T ₈ (60')	8.50	4.67	93	5.01	2491	223	42	0.159	0.700	0.158	14.8	4.70	431	1839	11949	3049	-558	158

Dog 12, female; 0.531 sq. m. surface area

Control Average (3 x 20')		0.87	66	1.28	233	28	0.0069	0.006	0.145	9.87	0.06	616	4240	512	59
T ₁ (20')	5.30	7.56	75	1.00	08	34	0.120	0.090	0.150	11.6	0.83	742	4752	512	59
T ₂ (20')	5.30	7.56	4.87	97	5.02	106	0.205	1.300	0.104	16.5	6.30	842	5028	788	54
T ₃ (20')	5.30	7.56	13.11	120	10.02	-57	0.183	2.40	0.170	21.1	12.00	204	5079	839	51
T ₄ (20')	5.30	7.56	17.44	139	12.58	-294	0.201	3.50	0.186	27.0	13.60	348	5179	939	54
T ₅ (20')	5.30	7.56	17.44	139	12.58	-294	0.183	3.20	0.186	30.3	11.00	402	5469	1229	52
T ₆ (20')	5.30	7.56	17.44	140	12.46	-757	0.207	3.60	0.186	27.3	13.80	465	5781	1541	53
T ₇ (20')	5.30	7.56	8.10	68	11.91	-806	0.210	1.70	0.188	13.3	13.00	555	6176	1036	55
T ₈ (20')	5.30	7.56	10.13	105	9.62	-893	0.207	2.10	0.193	21.1	8.70	644	6368	2158	51
T ₉ (20')	5.30	7.56	11.86	109	10.91	-972	0.204	2.10	0.204	23.0					50
T ₁₀ (20')	5.30	7.56	9.30	90	11.05	-1055	0.210	19.6	0.210	19.6					47

A comparison of the sodium and water rejection fractions showed them to be correlated (fig. 3). During control periods the rejection fraction for water exceeded that for sodium several fold. When infusion of the test solution was begun both fractions increased. The sodium rejection fraction, however, rose relatively more rapidly to reach a limiting value approximately equal to that for water. When this limiting value had been attained, further increases in the sodium and water rejection fractions were linearly related regardless of the concentration of the test solution infused or the magnitude of sodium excretion.

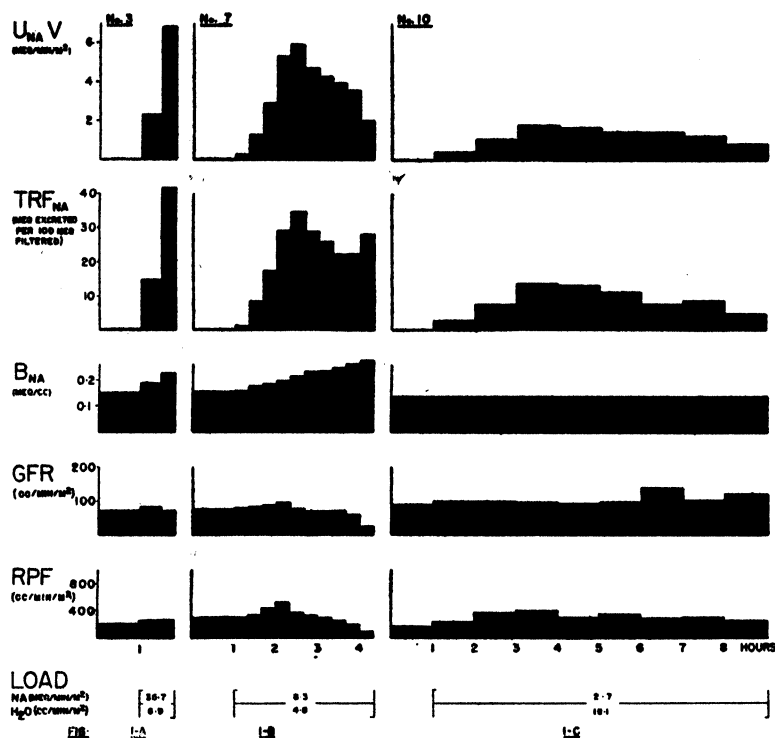


Fig. 1a, b, c. RELATION OF SODIUM EXCRETION TO sodium tubular rejection fraction, plasma sodium concentration, glomerular filtration rate and renal plasma flow under varying conditions of sodium load.

By definition, the relation between the sodium and water rejection fractions should be reflected in the ratio of the corresponding urine and plasma sodium concentrations.³ A comparison of the two latter parameters over the entire group of experiments (fig. 4) showed that the sodium concentration in the urine rose with infusion of the test solution to reach a limiting value which approximated the plasma concentration. Thereafter a further increase in urinary sodium concentration was obtained only in the presence of an equivalent rise in the plasma sodium level.

$$^3 TRF_{Na} = \frac{U_{Na} \cdot V}{GFR \times B_{Na}} \times 100; \text{ and: } TRF_{H_2O} = \frac{V}{GFR} \times 100; \text{ whence: } \frac{TRF_{Na}}{TRF_{H_2O}} = \frac{U_{Na}}{B_{Na}}.$$

DETERMINANTS OF SODIUM EXCRETION RATE

A number of associated factors were studied in an effort to determine the intermediate steps by which sodium administration provoked a rise in sodium excretion:

Plasma Sodium Concentration. No consistent relationship was established between sodium excretory rate and plasma sodium concentration. Infusion of isotonic solution produced marked increases in sodium excretion with little alteration in plasma sodium level (fig. 1 C). Comparisons between animals to which hypertonic solutions were administered failed to demonstrate a correlation between the rate of sodium excretion and the plasma sodium level. Serial observations in the same

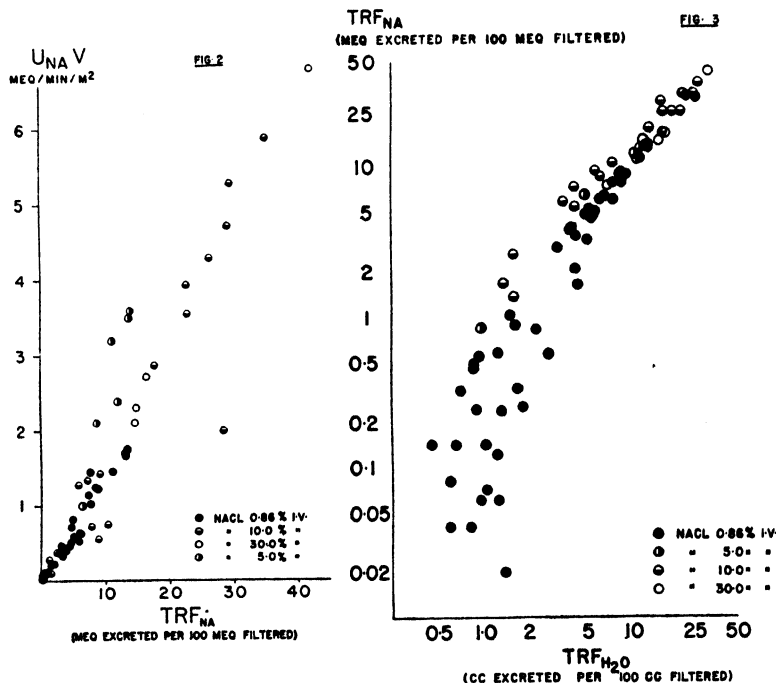


Fig. 2. RELATION OF SODIUM EXCRETION to sodium tubular rejection fraction.

Fig. 3. RELATION OF SODIUM AND WATER tubular rejection fractions under sodium load.

animal showed that the maximum sodium excretory rate often developed prior to the attainment of the maximum plasma concentration. In such instances, the sodium output entered a declining phase while the plasma sodium level was still rising (fig. 1 B, table 1, animals 6, 7, 12).

Sodium Balance, Extracellular Sodium Mass and the Sodium Space. Three successive measurements of the basal extracellular fluid volume were made during the control periods by the mannitol technique (5). The average of these values was used to calculate the basal quantity of extracellular sodium by means of the expression:

$$XC_{Na} = XCV \times B_{Na}$$

Where: XC_{Na} = Extracellular sodium mass (mEq/m²);

XCV = Extracellular fluid volume (cc/m²);

B_{Na} = Plasma sodium concentration (mEq/cc.).

The sum of the basal extracellular sodium mass and the net sodium exchange at the end of any clearance period subsequent to the control period was taken to represent the total extracellular sodium at that time. This latter figure was divided by the corresponding plasma sodium concentration to estimate the volume of the sodium space, assuming uniform extracellular distribution of the infused sodium.

On the basis of these computations, it appeared that the continuous administration of sodium chloride solution, regardless of rate or concentration, resulted in a progressive increase in the values for extracellular sodium mass and sodium space during periods of observation up to 10 hours (table 1, columns 16, 17).

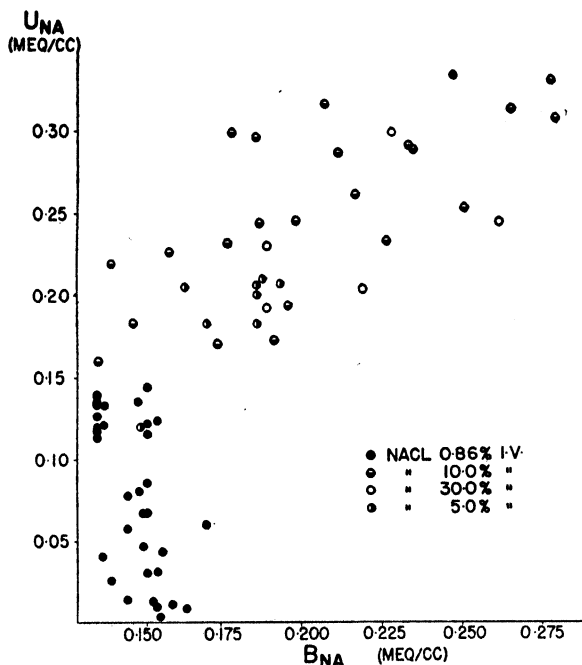


Fig. 4. RELATION OF SODIUM CONCENTRATIONS in urine and plasma under varying conditions of sodium load.

The maximal rate of sodium excretion was not proportional to the sodium balance, the extracellular sodium or the sodium space, either in the same animal or in comparisons between animals. Low rates of sodium excretion were often found in the presence of a markedly positive sodium balance, suggesting that the increase in this and related quantities (extracellular sodium mass and sodium space) rather than constituting a stimulus to sodium excretion was instead the consequence of failure by the animal to respond to sodium loading by an adequate increase in renal excretion. Comparison of these factors in animals infused at comparable rates of sodium and water administration (fig. 5) showed that the animal which developed the smaller sodium excretory rate manifested the larger increment in sodium balance, extracellular sodium mass and sodium space.

Water Balance. Administration of hypertonic solutions was followed almost

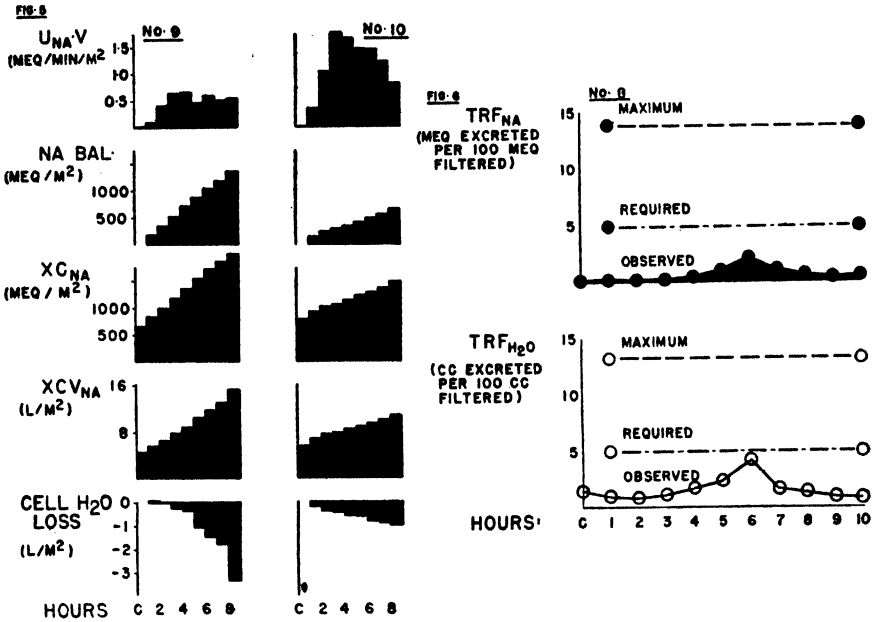


Fig. 5. INVERSE RELATION of sodium excretion to sodium balance and associated factors, where: $U_{Na} \cdot V$ = Sodium excretion; Na Bal. = Net sodium exchange; XC_{Na} = Extracellular sodium mass; XCV_{Na} = Extracellular sodium space; Cell H₂O loss = Cumulative cellular dehydration.

Fig. 6. COMPARISON OF TUBULAR rejection fractions observed during infusion of 0.86% NaCl at the rate of 0.6 mEq/min/m² with the rejection fractions which would have been required for maintenance of sodium and water balance. Indicated maximum rejection fractions were measured in other animals infused with the same sodium concentration at higher rates.

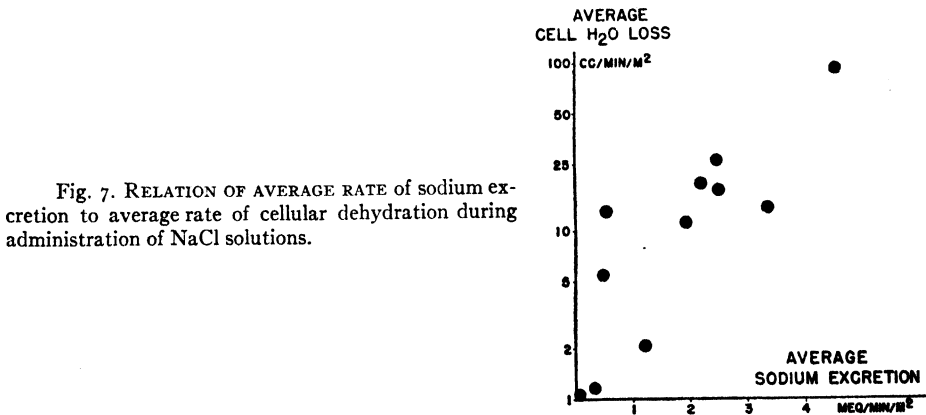


Fig. 7. RELATION OF AVERAGE RATE of sodium excretion to average rate of cellular dehydration during administration of NaCl solutions.

uniformly by a negative water balance, while isotonic solutions produced a positive balance (table 1, column 7). No correlations were established between the rate of sodium excretion and the direction or magnitude of the net water gain or loss.

Hematocrit. Serial hematocrit values were determined on 6 animals as an index of blood volume change. Administration of the test solution was followed by a fall in

hematocrit value, succeeded in some instances by a secondary rise (table 1, animals 7, 9, 10). In all but one case the minimum level coincided with or followed the period of maximum sodium output. Beyond this no correlation between the two factors was established.

Cellular Dehydration and Osmotic Balance. In the majority of animals observed for periods greater than 3 hours the rise in sodium excretory rate was succeeded by a decline in output despite an increasing distortion of the sodium balance and its associated factors (table 1, animals 7, 8, 9, 10, 12). The secondary decline in sodium output did not appear to be a consequence of the exhaustion of the animal's mechanism for sodium excretion, for it occurred even at low sodium injection rates during

which blood pressure, filtration rate and plasma flow were maintained or increased, and the maximum rate of excretion obtained was well below the maximum capacity for excretion as judged by the response of other animals to higher rates of sodium infusion at isotonic concentrations (fig. 6). The pattern of this response suggested the possibility that the initial rise in sodium excretion was due to a disturbance of osmotic equilibrium, while the secondary fall reflected a subsequent return toward equilibrium despite continued infusion.

Increased sodium output has been demonstrated to follow the subjection of osmoreceptor cells to concentrated solutions of sodium chloride injected into the

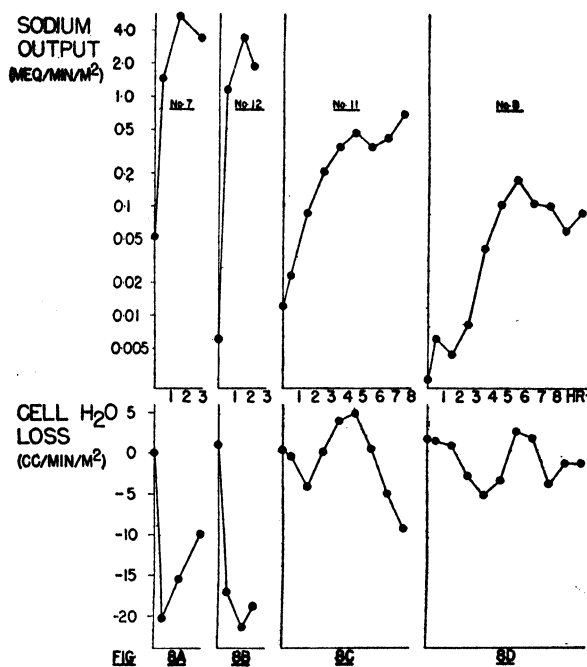


FIG. 8. RELATION OF SODIUM OUTPUT to serial changes in cellular dehydration rate during continuous infusions of NaCl solutions.

carotid artery (6). Since long-term (40-minute) infusions of dextrose of the same osmolarity were without effect, it appears likely that the stimulus to osmoreceptor cells depends on exposure to hypertonic concentrations of a non-permeating solute, resulting in osmotic pressure differences productive of cellular dehydration. Under these circumstances the rate of cell water loss becomes a measure of the osmotic pressure difference on the two sides of the cell membrane.

In the present experiments a mechanism for cellular dehydration was obvious in animals to which hypertonic solutions were administered. In all animals, however, an endogenous source of dehydration was presented, regardless of infusion concentra-

tion, by the continued secretion of a urine with a lower sodium concentration than plasma for varying periods after the start of the infusion (table 1, columns 10, 12). The operation of these factors was evidenced by the finding that the net change in sodium space was uniformly greater than the net water exchange, and could have been accomplished only by withdrawal of water from cells, barring a change in permeability toward sodium (table 1, columns 7, 19).

When the rapidity of cellular dehydration was computed by dividing the cell water change at the end of each period by the time over which it had occurred it was found that the average rates of sodium excretion and cellular dehydration were correlated ($r = 0.788$; fig. 7). A causal relationship was suggested by the temporal association of the two factors (fig. 8). Cellular dehydration was most intense in the early phases of the experiment. In 7 animals the maximum rate of cellular dehydration preceded the maximum rise in sodium excretion by an interval which ranged from 20 minutes to 3 hours. As sodium output increased the rate of cell water loss tended to fall. In some instances the magnitude of this reversal was sufficient not only to prevent further dehydration but to return water to cells (fig. 8 c & d).

In those animals in which the reversion of the rate of cell water loss was marked, a reduction in sodium output followed, regardless of the net sodium balance (fig. 8). When the infusion was continued for a sufficiently long period, this fall in sodium output was followed by a new cycle of increased cellular dehydration rate followed by a secondary rise in sodium output (fig. 8 c, 8 d).

Interpreting these results, it would seem that changes in sodium excretion are invoked by differences between the extracellular and intracellular osmotic pressures and persist only as long as such differences are maintained.

SUMMARY

The alterations in renal function which followed the administration of sodium chloride solutions were measured in a series of normal dogs. The resultant rise in sodium excretion was found due almost entirely to a decrease in its tubular reabsorption, and was accompanied by a correlated decrease in the reabsorption of water. Renal plasma flow was uniformly increased. No consistent change in glomerular filtration rate was demonstrated.

Increased sodium intake appeared to influence sodium excretion primarily by establishing an osmotic pressure difference between extra- and intracellular fluids, as estimated by the rate of cell water loss. Resolution of such osmotic pressure differences, as judged by a decline in the cellular dehydration rate, was followed by a fall in sodium excretion regardless of the magnitude of the plasma sodium concentration, sodium balance, extracellular sodium mass or sodium space. The data suggest that the major determinant in sodium regulation is not the preservation of sodium balance but the maintenance of osmotic homeostasis.

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ALTERATION OF NEURON EXCITABILITY BY RETROGRADE DEGENERATION¹

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THE definition and understanding of the altered physiological activity of the chromatolysed, degenerating and diseased nerve cell are fundamental problems in neurology. It is essential that the well-known pathological pictures of nerve cells be correlated with their functional concomitants before the symptomatology and recovery processes from neurological disorders, acute and chronic, can be understood. In addition, the problem of the nature and function of cytological structures depends to a great extent on the use of experimental alterations of cellular elements as a tool in linking anatomical entities with specific functions.

It is only natural that chromatolysis, the experimental reaction most easily controlled, should be the first cell alteration to be subjected to this analysis. Acheson, Lee and Morison (1) cut the phrenic nerve and noted a change in the activity of the motor cells, during the respiratory cycle, which roughly paralleled the usual period of chromatolysis and was for the most part a deficiency in cell firing. The loss of the proprioceptive or two-neuron reflex during the chromatolytic cycle of the motor neurons has been described (2) and it has been demonstrated that chromatolytic dorsal root ganglion cells show no loss of conductile properties (3) and that the antidromic cell potential is decreased during the chromatolytic period (4). These facts indicate that the interference of cell function occurs at the cell body level and involves neither the sensory side of the reflex arc nor the s, naptic apparatus.

The theory that the physiological effect of chromatolysis is on one general threshold of irritability of the cell rather than on the mechanisms of specific reflex pathways, as the evidence cited above indicates, leads to interesting consequences. One is that any reflex such as the tibial-peroneal reflex of Bernhard (5) will serve as a test for the general phenomenon. Any result from inspection of the effects of chromatolysis on this reflex which differ from those mentioned above would render the theory untenable. Strengthening of the theory, however, which would result from the demonstration of the predicted results in the instance of such an indirect reflex would require an inspection of the current theory of neuron activation.

A further consequence of the theory that the threshold of excitability is responsive to the axon reaction is that chromatolysed nuclei would show certain predictable changes in the relative numbers of cells falling into the subliminal fringe upon the application of single afferent stimuli. As the threshold of the neurons of the nucleus declines, the fraction of cells excited subliminally would increase with respect to those raised above threshold. However, as the rate of decreased excitability wanes, the original fraction value might well be re-established.

METHODS AND RESULTS

Cats and rabbits were used in the experiments. Sectioning of the combined tibial and peroneal nerve on the right side at mid-thigh level was done with aseptic

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precautions under nembutal anesthesia. After a suitable recovery period, anesthesia was again produced with dial or nembutal and the reflex activity tested on both the affected right side and on the normal left side which served as control. A three-channel sweep-synchronized stimulator, condenser coupled amplifier, and cathode-ray oscillograph were used for the physiological testing. The spinal cords were fixed in formalin, sectioned and stained with cresyl-violet and erythrosin for study of chromatolysis.

TABLE 1. EFFECTS OF AXON SECTION ON TIBIAL-PERONEAL REFLEX OF CATS

CAT NO.	DAYS DE-GENERATED	INCREASED LATENCY, MSEC.	FORM AND SIZE	CHROMATOLYSIS TYPE
1	2	0.3	Normal form, size	1
2	3	0.5	Normal form, reduced	2
3	4	0	Simplified, reduced	2
4	5	1.2	" "	2
5	6	0.3	" "	2
6	7	0	" "	2
7	8	1.8	" "	2
8	8	0	" "	2
9	9	0.2	Normal form, size	2
10	9	0	Simplified, reduced	*
11	9	2.0	" "	*
12	10	0.3	" "	2
13	12	1.1	Normal form, size	2
14	12		Absent	*
15	13		"	2
16	13	3.6	Simplified, reduced	*
17	13		Absent	2
18	14		"	*
19	15		"	2
20	18	3.0	Simplified, reduced	2
21	27	2.8	" "	3
22	27	2.1	" "	3
23	34	0.5	Simplified, normal size	3
24	35	1.5	Simplified, reduced	2
25	102	4.8	" "	*

* No Nissl studies made.

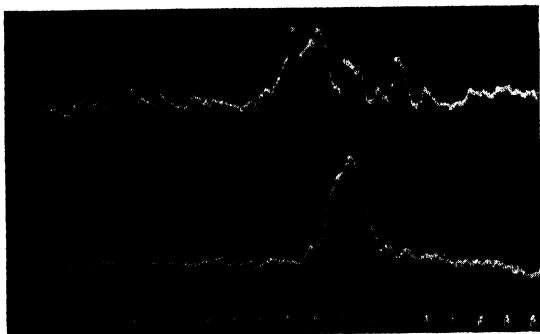
A series of twenty-five cats was studied (table 1). The days of retrograde degeneration varied from 2 to 102 days, with most careful coverage on the first two weeks. The changes noticed in this series were of four types. There was an increased latency of the response of the peroneal nerve following tibial stimulation. This was seen in every animal from 9 days on as well as in 4 of 8 which were tested earlier. Only one animal, at two days, showed the reverse trend; three others, at 3, 6 and 8 days, showed no change. In the later experiments, significant delays were found, ranging up to 3.6 msec. Interpretation of the early half of the series is complicated by the fact that the latency difference between the two sides is apparently of the order of magnitude of the variability of the preparation. Attempts were made to

insure equal conduction distance on the left (normal) and right (chromatolysed) side but these were not always successful. The errors introduced by this factor appear to range up to 0.5 msec. No interpretation is attempted on the 2-day animals, where a decreased latency appeared.

The second effect shown in this series was an abolition of the reflex completely during the height of the degenerative cycle. This occurred in 5 animals, at 12, 12, 14, 15 and 26 days. All animals of this period do not show this loss, but those which do not show the complete loss show very extended latencies.

The third effect noted is the simplification of the form of the reflex curve. Figure 1 shows a typical example in which the normal reflex pattern—usually a slowly decreasing multimodal curve exhibiting three, four, or even five definite peaks of activity—was altered on the chromatolysed side to produce a much simpler curve. The dispersal of the normal activity strongly indicates repetitive firing of the motor neurons. We are led, thus, to interpret the simplification as shown in figure 1 as an indication of the shortening of the train of impulses generated by the individual

Fig. 1. ALTERATION OF REFLEX RETURN on peroneal nerve of cat in response to tibial stimulation on normal (a) and chromatolysed (b) sides. The duration of the retrograde degeneration was 35 days.



motor cell. Through some mechanism which is not obvious, synchrony of the initial discharge sometimes characterizes the altered response with the curious result that the amplitude of the response as measured by peak voltage is actually increased (see below).

A fourth consequence of the chromatolysis was found to be a decrease in the total reflex outflow. Because of the tendency for synchrony as mentioned above, the height of the reflex record on the affected side was occasionally increased, but even in such cases a decreased return was obvious. Because of the dispersion of this particular reflex, peak voltage is a poor measure of the actual response. We have used, instead, the area under the potential curve as a measurement of the total activity. Where strong synchrony and no repetitive firing are the rule, as in the proprioceptive reflex, close correlation must exist between the height of the response and the number of units active. With monophasic recording, the same correlation would obtain between the number of conducted impulses and the areal summation of the curve. We may conclude, thus, that the activity as measured in impulses is decreased on the chromatolysed side.

In summary, it is to be seen that the four functional alterations observable in the chromatolysed motor nuclei of these cats are obviously the result of a dysfunction

which, acting to decrease excitability, makes the reflex return to a supramaximal (for A fibers) shock to the tibial nerve later, simpler, smaller and occasionally absent.

Study of stained sections of the spinal cords showed chromatolysis in the earliest member of the series (two days) and apparent in all in which Nissl studies were made. The classification proposed by Campbell and Novick (6) of the stages of axon reaction was used to record the degree of chromatolysis seen in the sections. However, no quantitative expression was attempted because it was not feasible with the material on hand to attempt to circumscribe the nuclei involved. The numerical stages indicated in table 1 represent the most prominent class of altered cells in the sections sampled. These findings agree essentially with those of Barr and Hamilton (7), who have studied the time course of the axon reaction in the cat using similar categories.

TABLE 2. CONDITIONING EFFECT OF TWO STIMULI IN RABBITS

RABBIT NO.	DAYS OF DEGENERATION	AVERAGE AREA OF TEST VOLLEY EXPERIMENTAL NORMAL	INCREASED LATENCY MSEC.	MAX. AREA OF CONDITIONED RESPONSE AS % OF 2X TEST RESPONSE	
				Normal	Experimental
1	3	33%		84	120
2	3	51		79	101
3	5	12	.07	100	165
4	5	40	.55	95	135
5	5	103	.60	95	155
6	5	11	.21	85	155
7	8	21	.54	65	98
8	8	51	.50	80	80
9	8	13	.33	75	155
10	11	24	.50	80	70
11	12	17	.74	79	79
12	12	64	.62	100	79
13	15	78	-.2	76	58
14	16	61	1.55	109	83

Some mention should be made of the fact that the classifications mentioned above do not serve adequately for study of the recovering cell. It would appear that the later stages of the chromatolytic cycle do not merely retrace the rising phase. Thus, the designation of the cells of *cat 24* as being in the second stage is provisional only.

In similar experiments in a series of 14 rabbits, in addition to the observation of the alteration of the simple reflex, a systematic study was made of the conditioning curves obtained by two shock stimulation. Table 2 shows the data obtained. Retrograde degeneration was studied from 3 to 16 days' duration. In none of the rabbits was there a complete obliteration of the reflex on the degenerated side as had been seen in some of the cats. The decrease in the reflex noted in the former series was similarly revealed in the rabbits (column 3, table 2). With the exception of one animal, there was a considerable decrease in the area under the potential curve of the returned reflex on the affected side. Latency was increased from 0.3 to 1.5 msec., with but one exception Figure 2 illustrates a typical experiment and the simplification of the pattern on the right side conforms with the rule.

The analysis of the conditioning curves of the rabbit series reveals an additional

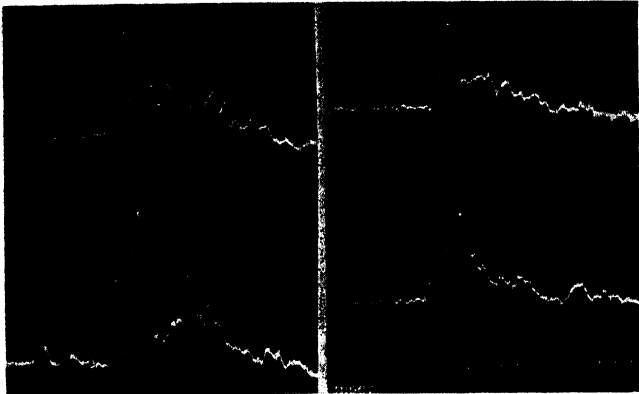


Fig. 2. ALTERATION OF REFLEX RETURN on peroneal nerve of rabbit in response to single and double stimuli. Left side of figure shows normal side, right shows experimental side. The duration of degeneration was 3 days.

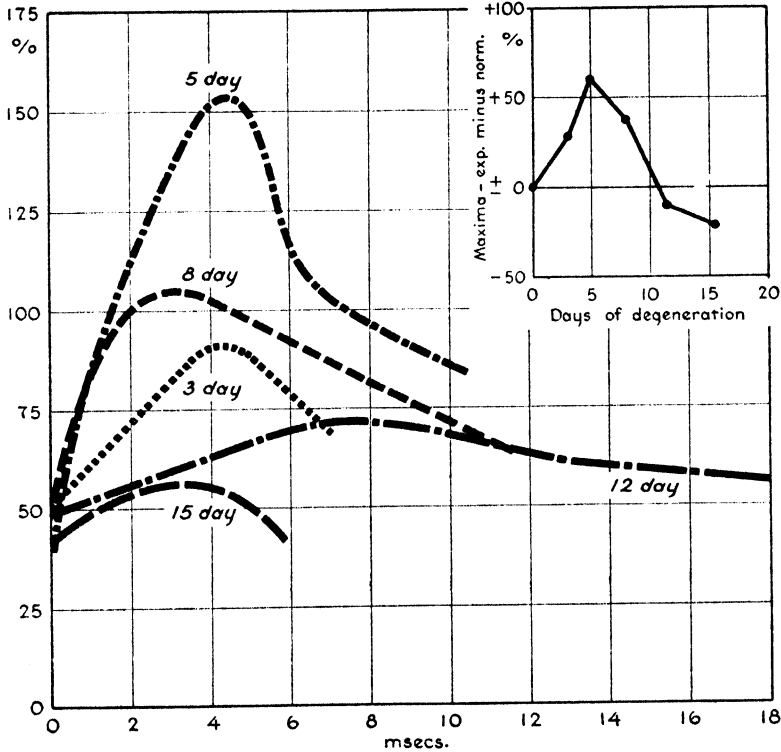


Fig. 3. CONDITIONING CURVE OF REPRESENTATIVE RABBITS SHOWING conditioning curves of degenerated side in percentage of twice the area under the test potential curve. *Insert.* Comparison of degenerated side with normal plotted according to durations of retrograde degeneration.

sign of threshold involvement on the part of the final common pathway neurons. The second of two supramaximal shocks produces a reflex on the experimental side which greatly differs from that on the control side, (fig. 3). This difference varies

with the duration of the axon section. In column 5 of table 2 the maximal electrical activity attained by two successive supramaximal shocks is listed as a percentage of twice the activity of the test shock. The differences are obtained by subtracting the figure for the normal side from that of the degenerated side. These figures are averaged for the various days of degeneration and plotted as a function of time in figure 3 (insert). Even though the relative percentage values of the abnormal side may exceed its control at short degenerative intervals the absolute values of total electrical activity are always much less on the experimental side.

The curve of conditioned response to two supramaximal shocks, (fig. 3), may be taken as a profile of the subliminal fringe excitability, with the added factor, especially in the very short intervals, of the refractory periods of the various portions of the reflex arc. The maximum of the curve, which is to be found at the interval of several milliseconds, is probably only insignificantly more correlated with any other factor than the excitability of the subliminal fringe. Thus we may interpret the results shown in column 6, table 2 and figure 3 as indicating a large (percentagewise) subliminal fringe in the early stages of this series, declining to below normal in the 16-day group. Associated as this is with a decreased reflex return to a single shock, this then may be interpreted as increased threshold of the member cells of the nucleus stimulated. With the slight increase of threshold in the early series, a large percentage of those failing to respond to the first stimulus were available to the second. With the more profound decrease in excitability, relatively fewer were left with a residuum of activity after the first stimulus sufficient to help achieve effective threshold as a result of the second.

DISCUSSION

The axon reaction is known by several morphological criteria. Cells with axons severed show a cycle of degenerative changes in the cell body. Best known of these are the fragmentation and the pulverising of the Nissl granules and the alterations in the position of the cell nucleus. Increased volume of the cell, altered enzyme concentrations and nuclear cap formation are also known. It cannot be assumed that all or any of these phenomena are directly correlated with the altered physiology. For the present, however, we must consider the mechanics of neuronal excitation in the light of current models of the origin of the conducted impulse and some refinement of these ideas may be devised from the data offered above.

The simplest model of the reflex arc will not suffice here. Simple all-or-none transmission over the hypothetical synapse would be insufficient to explain the graded nature of the changes of response associated with retrograde degeneration of the nerve cell. A local graded response at the level of the neuron affected must be assumed and two current models, each the product of many minds, may be examined. The first is based upon a consideration of the external electrical fields produced by resting and active neurons, and on the effects upon the cells of polarizing currents. In a paper discussing galvanotaxis in the crayfish, Loeb and Maxwell (8) presented a diagram from which nearly all of the more recent refinements of the theory may immediately be deduced but a thorough exposition is lacking in the accompanying text. Forbes (9), Barron and Matthews (10), Gerard and Libet (11) and Gesell (12)

have developed the model; the last-named author contributing a discussion of the idea that the conducted impulse is generated at the axon hillock. Briefly, a cell is postulated which is organized in such a way that its metabolic activity is reflected in an electrical bipolar field involving current flow both within and external to the cell. The field is assumed to be oriented with the axis of the neuron so that in steady states a certain current density exists at the membrane of the axon hillock, which serves as a pulse-signal generator in that the conducted impulses arise at this point and correspond, in frequency, to the flux. An alteration of the external field, as induced by the explosive negativity of the boutons, or from any other phenomenon will effect the current density at the axon hillock and transmit signal by modulating the frequency of the generation of conducted impulses. It should be remarked here that the ventral horn cells would seem to exist in a steady state of zero frequency of the conducted impulses, at least in the experimental situation described in the paper. The current alternative to the bipolar model discussed above is an evolutionary product of the Lucas dictum (13) and has been best described in the analysis of Lorente de Nó (14). In this scheme of neuron activity, signal is transmitted by conduction of active states from the boutons or other perineuronal endings of specific telaxons across a physiologically defined gap, the synapse, to excite conducted waves of depolarization on the cell membrane of the body and/or dendrites of the secondary neuron. Graded correlation of afferent and efferent activity may be achieved by the operation of local responses, a function of the secondary cell, which by varying with the state of the cell metabolism (hence polarity of membranes) would show altered spatial and temporal summation. The ability of the local responses to achieve the magnitude necessary for the local generation of a conducted impulse would be graded, under experimental conditions such as considered in this paper, to give results such as actually obtain. Between these two models we may not conclusively choose on the basis of the present data. In the absence of a *tertium quid*, the only slight contribution possible is to remark that the morphologic variants with this cycle of altered response affect the internal structure of the cell and not at least in a way to be observed at the present time, the cell membrane. From this some slight preference to the bipolar model may be expressed.

The cytological alteration studied here is 'unphysiological' in the sense that axon destruction is rarely if ever of occurrence in the normal animal. There is no reason to think that this particular type of degeneration plays a part in the economy of the animal. Yet there is a possibility, supported by a small but insistent literature, that cytological alteration similar to this type of chromatolysis may result from over-activity of nerve cells. Barr and Hamilton (7) found slight chromatolysis in over 10 per cent of the motor cells of their control spinal cords. If this should prove well-founded, the importance of these changes in the irritability of the cell might well have to be reckoned as having some function in the integrative process.

SUMMARY

A study was made of the tibial-peroneal reflex of cats and rabbits following intervals of retrograde degeneration of the primary motor neurones of the peroneal nerve of 1 to 102 days.

Chromatolysis is associated with an alteration of the reflex activity of the spinal motor nuclei. The tibial-peroneal reflex, during the cycle of retrograde degeneration shows a) an increase in latency, b) an occasional complete cessation of reflex transmission, c) simplification of pattern of reflex when present, and a decrease in the activity of the involved neurons as measured by the area under the curve of the efferent potential. Conditioning studies indicate that there is a relative increase, during the rising phase of the cycle, of the subliminal fringe which is a measure of the increased threshold of the cells.

The findings are discussed in the light of current models of the neuron.

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EFFECT OF 2-METHYL NAPHTHOQUINONE ON THE ACTION POTENTIAL OF NERVE AND MUSCLE

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THE effect on the action potential of nerve and muscle of a potent and probably specific inhibitor (1-3) (2-methyl naphthoquinone) of choline acetylase was investigated to ascertain whether or not it impairs the function of nerve and muscle. Should impaired function be exhibited, and, assuming that disturbances in acetylcholine synthesis under the circumstances is the only biochemical action of the naphthoquinone, it might be inferred that acetylcholine is essential to the maintenance of proper neuro-muscular function.

METHOD

Action potential measurements were made on *a*) isolated frog organs and *b*) organs of mouse and rat *in situ*. Over 20 preparations were used in each group.

A. Isolated Frog Organs. Either the sciatic nerve or the sciatic nerve together with the attached gastrocnemius muscle were suspended in frog Ringer's solution at room temperature (23° C.). During the entire experiments a gas mixture containing 5 per cent carbon dioxide and 95 per cent oxygen was bubbled through the bathing fluid.

B. Organs in Situ. The spinal cords of mouse and rat were destroyed up to the midthoracic region under light ether anesthesia. A few hours afterwards the animal and its leg at the knee joint were firmly fixed to an animal board.

Action Potential Measurements. Stimulating bakelite insulated silver wire electrodes, 4 mm. apart, were placed under the nerve. The same type of recording electrodes were placed under the nerve (2 cm. apart) and in the muscle (one in the tendon of the gastrocnemius muscle and the other in the upper half of the muscle). Stimuli with a repetition rate of 11 pulses per second and of 'supramaximal' intensity were delivered every 10 minutes for 30 seconds. Supramaximal intensity was maintained by increasing the intensity of the stimulus wherever the threshold increased. The action potentials of the nerve and muscle (either diphasic or monophasic) were observed by a cathode ray oscilloscope. The sweep circuit of the oscilloscope was synchronized with the stimulator so that successive stimuli and action potentials were superimposed on the screen of the cathode ray tube. Either single or superimposed action potentials were photographically recorded.

2-Methyl Naphthoquinone. After the action potential remained constant for two to three series of stimulations the effect of the naphthoquinone was tested by the following method: *a*) The isolated organs were immersed during the 10-minute rest periods into a Ringer's solution containing the naphthoquinone in concentrations of $1 \times 10^{-5}M$; *b*) the animals were injected with the naphthoquinone intraperitoneally in amounts computed to give a final body concentration of $1 \times 10^{-5}M$.

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Direct Stimulation of Muscle. The muscle was directly stimulated with a current having a repetition rate of 5 pulses per second and being of 'supramaximal' intensity. This direct stimulation was delivered before and immediately after repetitive indirect stimulation. The muscle contraction was recorded by a kymograph through an isotonic lever attached to the end of the tendon of the gastrocnemius muscle.

Controls. Isolated preparations and living animals treated and stimulated as described above (except that they were not treated with the naphthoquinone) served as controls.

RESULTS

A, 1. Isolated Nerve Preparation. The nerve was stimulated for 30 seconds at 10-minute intervals. The threshold, the amplitude of the first action potential, as well as the conduction velocity, of the control nerves remained unaltered for several

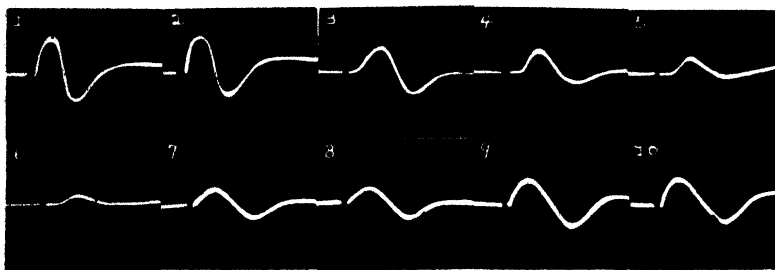


Fig. 1. ACTION POTENTIAL RECORDS OF ISOLATED NERVE (stimulation with 11 pulses per sec.). 1) Action potential taken at the beginning of 30-sec. stimulation period, nerve immersed in Ringer's solution; 2) action potential taken at the end of the 30-sec. stimulation period; 3) same as 1 taken after 1-hour immersion in the solution of the naphthoquinone; 4) same as 2 taken after 1-hour immersion in the solution of the naphthoquinone; 5) same as 1 taken after 1½-hour immersion in the solution of the naphthoquinone; 6) same as 2 taken after 1½-hour immersion in the solution of the naphthoquinone; 7) same as 1 taken after 20-min. washing with Ringer's solution; 8) same as 2 taken after 20-min. washing with Ringer's solution; 9) same as 1 taken after 40 min. washing with Ringer's solution; 10) same as 2 taken after 40-min. washing with Ringer's solution.

hours. The amplitude of the action potential decreased on the average 5 per cent during each 30-second stimulation period. Complete recovery occurred during the 10-minute rest periods.

The conduction velocity and the amplitude of the action potential of the nerve decreased and the threshold increased when immersed in solutions of the naphthoquinone. An immersion of 45 minutes or more was required before the appearance of the first changes in nerve function. The amplitude of the action potential decreased on the average 30 per cent during each 30-second stimulation period. Recovery did not occur during the 10-minute rest periods in the naphthoquinone solution (fig. 1). Finally, the action potential disappeared almost completely. Prolonged washing with Ringer's solution reversed the effect of the naphthoquinone. Recovery of the conduction velocity preceded the recovery of the amplitude of the action potential.

2. Isolated Nerve-Muscle Preparation. The threshold and the interval of time elapsing between the stimulating shock and the appearance of the muscle action potential, with nerve-muscle preparations immersed only in Ringer's solution, remained unaltered for at least one hour. The amplitude of the muscle action potential usually increased during the first few seconds of stimulation and did not decrease during the

30-second stimulation period as compared to the first action potential of this stimulation period. The amplitude of the first action potential of each 30-second stimulation period remained unaltered for at least half an hour and decreased on the average by 20 per cent at the end of one hour.

Immersion of nerve-muscle preparations in solutions of the naphthoquinone caused within a few minutes an increase in the latency and the threshold, and a decrease in the amplitude of the action potential. The amplitude of the action potential decreased on the average 50 per cent during each 30-second stimulation period. Recovery did not occur during the 10-minute rest periods in the naphthoquinone

Fig. 2. ACTION POTENTIAL RECORDS OF ISOLATED GASTROCNEMIUS MUSCLE during stimulation of the sciatic nerve (stimulation with 11 pulses per sec.) 1) Action potential taken at the beginning of the 30-sec. stimulation period before immersion in the solution of the naphthoquinone; 2) action potential taken at the end of the 30-sec. stimulation period; 3) same as 1 taken after 10-min. immersion in the solution of naphthoquinone; 4) same as 2 taken after 10-min. immersion; 5) same as 1 taken after 30-min. immersion; 6) same as 2 taken after 30 min. immersion.

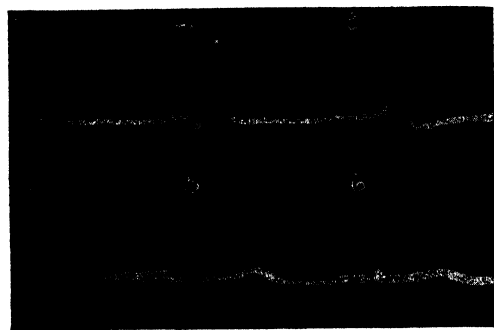
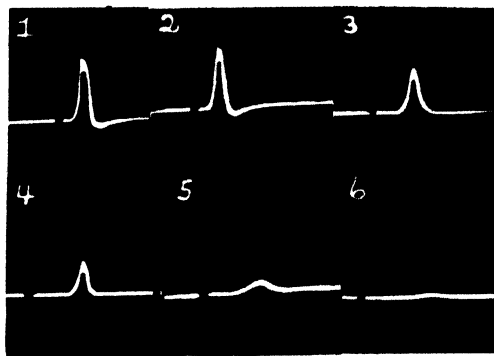


Fig. 3. ACTION POTENTIAL RECORDS OF GASTROCNEMIUS MUSCLE of rat *in situ* during indirect stimulation (stimulation with 11 pulses per sec.) 1) Action potential taken at the beginning of a 30-sec. stimulation period before injection of the naphthoquinone; 2) action potential taken at the end of the 30-sec. stimulation period; 3) same as 1 taken 10 min. after injection of naphthoquinone; 4) same as 2 taken 10 min. after injection; 5) same as 1 taken 30 min. after injection; 6) same as 2 taken 30 min. after injection.

solution. Within 30 minutes the amplitude of the first action potential of the 30-second stimulation period averaged 25 per cent of the amplitude of the corresponding first action potential before immersion in the naphthoquinone solution (fig. 2).

B, 1. Indirect Stimulation of Muscle in Situ. The threshold, the amplitude of muscle action potential, as well as the conduction velocity, remained unaltered in the control animals during indirect stimulation for several hours. The amplitude of the action potential decreased on the average 10 per cent during each 30-second stimulation period. Complete recovery occurred during the 10-minute rest periods.

In animals injected with the naphthoquinone the amplitude of the muscle action potential decreased and the latency and the threshold increased during indirect stimulation. The amplitude of the action potential decreased during each 30-second stimulation period on the average by 45 per cent. Recovery did not occur during the 10-minute rest periods. The action potential finally dropped to zero (fig. 3).

2. *Direct Stimulation of Muscle in Situ.* In the control animals the magnitude of contraction of the gastrocnemius muscle remained unchanged during repetitive indirect stimulation. The magnitude of contraction on repetitive direct stimulation before and after indirect stimulation, also remained the same.

In injected rats the contraction of the gastrocnemius muscle on repetitive indirect stimulation decreased in a degree similar to that of the action potential. The magnitude of muscle contraction on repetitive direct stimulation before and after indirect stimulation was approximately the same (fig. 4).

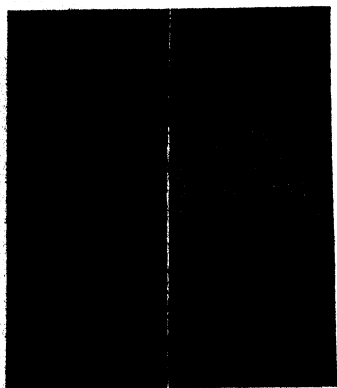


Fig. 4. KYMOGRAPH RECORDS OF THE GASTROCNEMIUS MUSCLE during direct stimulation (stimulation with 5 pulses per sec., rat). *Left*, record taken before indirect stimulation in a rat injected with the naphthoquinone. *Right*, record taken immediately after indirect stimulation.

DISCUSSION

The experiments indicate that 2-methyl naphthoquinone decreases the conduction velocity and the amplitude of action potential of nerve and increases the threshold. The contractile mechanism in the muscle is, however, not impaired in the presence of 2-methyl naphthoquinone in a concentration of approximately $1 \times 10^{-5}M$ since the muscle contraction on repetitive direct stimulation did not change significantly.

These results agree with those reported from this laboratory (4) showing that inhibitors of choline acetylase decrease the response of striated muscle to indirect stimulation as measured by myography. This decrease simulates a curare effect but is induced by a different mechanism. Curare interferes at the receptors of the effector cells with acetylcholine, whereas the naphthoquinone is a potent inhibitor of choline acetylase (1-3) and does not interfere, in the concentrations used, with acetylcholine at the receptors of the effector cells (1).

The decreased nerve and muscle function found in the presence of naphthoquinone in concentrations of $1 \times 10^{-5}M$ was probably due to its effect in decreasing acetylcholine synthesis, since the concentrations required to inhibit the activity of enzymes other than choline acetylase—cholinesterase (1), urease (5), catalase (6), papain (7), and enzymes involved in aerobic and anaerobic lactic acid formation (8)—are greater than $1 \times 10^{-5}M$. Other inhibitors of choline acetylase—monoiodoacetate, iodoacetate (9-11), toxin of *Clostridium botulinum* (12, 13), alloxan (4, 14-16), α - and β -naphthol (4, 14-16)—were also found to decrease the action potential (11, 4, 16). The results with many of these agents cannot, however, be interpreted as being ef-

fects only through inhibition of acetylcholine synthesis, e.g. iodoacetate and iodoacetamide inhibit, in the concentrations used, the metabolism of triosephosphate (17) causing a decrease of regeneration of energy-rich phosphate bonds. Therefore, iodoacetate and iodoacetamide probably act through many mechanisms besides inhibition of acetylcholine synthesis.

Acetylcholine probably acts at more than one point. It is, however, likely, that the anoxic depolarization of nerve is dependent on the presence of acetylcholine. This assumption may be made since *a*) anoxia induces depolarization of nerve (11) without an inhibition of the activity of choline acetylase (10); *b*) iodoacetate prevents anoxic depolarization (11) and inhibits acetylcholine synthesis (9, 10); and *c*) most of the other processes known that could be considered as the cause of anoxic depolarization (e.g. inhibition of glycolysis resulting in interrupted regeneration of adenosinetriphosphate) are inhibited in the nerve by both iodoacetate (17) and anoxia (11).

SUMMARY

The effect of 2-methyl naphthoquinone on the function of nerve and muscle was investigated *in vitro* and *in vivo*. In the presence of low concentrations of the naphthoquinone the latency and the threshold increased and the amplitude of the action potential of nerve and muscle decreased. The muscle function on direct stimulation was not significantly impaired. Since the naphthoquinone, in the concentrations used, is known to inhibit only the activity of choline acetylase, the dysfunction observed suggests that acetylcholine is necessary to maintain an optimal function of nerve.

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MUSCLE RECOVERY AFTER NERVE SECTION AND SUTURE

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THAT partially denervated skeletal muscle makes some spontaneous recovery, improving in weight and strength, was shown in three separate but almost simultaneous reports by van Harreveld (1), Hines, Wehrmacher and Thompson (2) and Weiss and Edds (3). The general experimental procedure used by these three groups was to remove a portion of the spinal roots supplying innervation to a skeletal muscle. There was then an early loss of weight and strength of the muscle followed by some spontaneous improvement. This improvement was proved not due to regrowth of the sectioned nerve roots. Rather it seemed to depend upon two factors: *a*) hypertrophy of the remaining innervated muscle and *b*) an increase in the number of innervated muscle fibers through adoption of some of the denervated muscle fibers by axone branches from the intact motor nerves. It seemed as if this second factor was the most important in the recovery.

It has long been suggested that by inducing increased branching of peripheral motor axones an improvement in partially denervated muscle could be obtained. Claims for such induced branching with good clinical results, have been made by Feiss (4) and Dogliotti (5). In these instances axone branching was produced by nerve section and suture or by nerve crush.

A much more extensive series of attempts to mobilize the residual nerve supply for reinnervation of partially denervated muscle was reported by Billig, van Harreveld and Wiersma (6). Their results did not show the spectacular success of Feiss (4) and of Dogliotti (5) but they concluded that nerve crushing (neurotripsy) by either an open or closed method did, in many instances, result in muscle improvement. This was particularly the case if the muscle had a poor innervation before neurotripsy was done. On the other hand, many of the better innervated muscles suffered a loss in size and function as a result of the neurotripsy procedure.

The question arises whether such a deliberate nerve section or crush will really enhance the tendency that intact fibers have for adoption of denervated muscle fibers. A recent report by Fredrick and Kossman (7) indicates that for the partially denervated anterior tibial muscle of the dog closed manual neurotripsy results in no increase of muscle weight and strength over that which will occur spontaneously.

In the experiments described here the question was posed as to whether the extent of recovery in a reversibly denervated muscle could be enhanced by neurotripsy. Rats were used as the experimental animal. A nerve lesion was made by cutting the sciatic nerve at the level of the greater trochanter and repair made by immediate end to end suture of the nerve. The nerve was cut with a fine scissors rather than with a knife on a block so that the end to end suture did not result in the best possible recovery. Statistically the method produces muscles which at maximum

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recovery have very similar degrees of partial denervation. Changes in muscle weight and muscle strength were followed for 158 days in such animals to determine the rate and extent of recovery. In similarly prepared animals a closed neurotomy was done on the gastrocnemius and the extent of muscle recovery compared with those without neurotomy. Further, a series of normally innervated muscles was subjected to closed neurotomy and the acute and chronic effect of this on fully innervated muscle so determined. The closed neurotomy was done by placing the leg of the animal upon a brass block and beating the gastrocnemius muscle through the skin with a small brass hammer driven by a 'Vibrotol'.

Muscle strength was measured with a torsion lever using optical recording. Stimuli were rectangular electrical pulses of supermaximal intensity and of a pulse duration and frequency suited to the irritability status of the nerve or muscle under test.

TABLE 1. MUSCLE CHANGES FOLLOWING NERVE SECTION AND SUTURE

NO. OF ANIMALS	TIME IN DAYS AFTER NERVE SECTION AND SUTURE	MUSCLE ¹					
		Weight	P ²	Strength			
				Direct stimulation		Nerve stimulation	
				Per muscle	P ²	Per muscle	P ²
6	0	97.7		97.0		113.0	
8	14	57.7	.000	28.1	.000	0.0	.000
8	28	36.8	.000	17.2	.014	1.1	.290
7	56	50.7	.000	20.3	.027	7.1	.001
5	82	70.5	.000	44.8	.000	30.4	.000
8	91	74.9	.239	61.5	.004	52.4	.000
9	158	75.3	.922	59.6	.768	57.1	.023

¹ Values expressed as percentage of the contralateral unoperated control. ² P for the Fisher t for successive differences.

Changes in muscle weight and strength in the time following nerve section and suture are shown in table 1. Weight recovery may be regarded as completed at 91 days after nerve section or about 63 days after the first functional signs of reinnervation. Permanent impairment has resulted from the nerve lesion as shown by the final weight attained.

The muscle response to direct stimulation lags behind the weight recovery. There is only a slight change in weight between the 82- and 91-day period while there is a significant increase in strength during the same time. This strength increase must be predicated on an improvement in the functional capacity of the muscle cell cytoplasm.

While the muscle strength recovery in response to direct stimulation seems to be complete at 91 days it will be noted that further increase in the muscle response to nerve stimulation occurs between the 91- and 158-day periods. From these relations in recovery it appears that the reinnervation has its first effect upon muscle weight, that functional restitution of the muscle cell cytoplasm follows this structural replacement, and that these increases in weight and strength, while dependent upon

the reestablished nerve supply, precede in time the actual achievement of motor control of the muscle by the nerve. Thus the sequence of restitution seems to detect a trophic influence of motor nerve upon muscle beyond the motor command that the nerve has over the muscle at that time.

The result of applying the neurotripsty technique to normally innervated muscle is shown in table 2. This indicates extensive acute damage to the muscle with motor nerve injury beyond the muscle damage. The gross appearance of such a muscle immediately after the beating is rather discouraging. Maserated, pulpy and bloody,

TABLE 2. MUSCLE CHANGES FOLLOWING NEUOTRIPSY TO COMPLETELY INNERVATED MUSCLE

NO. OF ANIMALS	TIME IN DAYS AFTER NEUOTRIPSY	MUSCLE ¹					
		Weight	P ²	Strength			
				Muscle stimulation		Nerve stimulation	
				Per muscle	P ²	Per muscle	P ²
6	0	101.3	.884	64.0	.027	44.0	.010
10	62	96.4	.392	99.7	.992	109.0	.300

¹ Values expressed as percentage of the contralateral unoperated control. ² P for the Fisher for difference from unoperated contralateral control.

TABLE 3. MUSCLE CHANGES FOLLOWING NEUOTRIPSY TO PARTIALLY INNERVATED MUSCLE

NO. OF ANIMALS	TIME IN DAYS FROM		MUSCLE ¹					
	Nerve section to neurotripsty	Nerve section to examination	Weight	P ²	Strength			
					Muscle stim.		Nerve stim.	
					Per muscle	P ²	Per muscle	P ²
5	25	82	103.0	.525	105.1	.506	116.2	.346
10	56	158	99.0	.936	102.2	.856	91.0	.191
8	91	158	101.7	.728	100.8	.922	93.6	.269

¹ Values expressed in percentage of average of muscles having same nerve cut, suture, and recovery time but no neurotripsty.

² P for Fisher t for difference between neurotripsty and non neurotripsty.

it appears that permanent damage must have been done. That these changes are completely reversible is shown by the response of such muscles after 62 days recovery. They are then the equal of their contralateral controls in all respects. From this it was concluded that such a neurotripsty could be done to produce over 50 per cent acute denervation and still allow complete recovery so that there should be no loss from the neurotripsty per se to mask any gain that might result from its application to partially denervated muscle.

The results of applying neurotripsty to partially denervated muscles are given in table 3. An early neurotripsty group was done 25 days after nerve section. At this time very little muscle reinnervation had occurred. An intermediate group received neurotripsty at 56 days, a time when very rapid reinnervation was occurring, and a late neurotripsty group was treated 91 days after nerve section when weight and

muscle strength recovery had reached a maximum. The results show that the neurotripsy procedure was without effect since it neither enhanced nor retarded the extent of recovery. Thus these results are in agreement with those of Fredrick and Kossman (7). The inducement of motor nerve fibers to adopt adjacent denervated muscle fibers is an attractive objective, but it appears that the neurotripsy procedure is not reliably adequate to induce motor nerve fibers to exceed their spontaneous adoption potential.

SUMMARY

The rate of restitution of muscle weight and strength after motor nerve section and suture has been determined. The sequence of the recovery of weight and of contractile power in response to direct stimulation and in response to nerve stimulation indicates a trophic influence of the motor nerve on the muscle beyond the ability that the nerve has for muscle excitation.

Neurotripsy done at various times after nerve section and suture did not alter the final extent of muscle recovery.

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RESPONSES ELICITED BY COMBINED STIMULATION OF PAIRS OF FIXED ELECTRODES IN THE UNANESTHETIZED MONKEY^{1,2}

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A NUMBER of reports have been made on the effects of stimulation of the cerebral cortex in the unanesthetized animal (1, 2) but we have found none concerning the effects of concurrent or immediately consecutive stimulation of two or more cortical points in such preparations despite the fact that such studies have been fruitful in the anesthetized animal. The effects of concurrent or successive stimulation of two points are of special interest in view of the problems raised by the discovery of the so-called suppressor bands (3, 4). The following report is concerned with the influence of stimulation of the anterior suppressor band upon the excitability of other cortical regions in the unanesthetized monkey.

METHODS AND RESULTS

Electrodes were implanted aseptically in 10 immature macaque monkeys under nembutal anesthesia (2). Figure 1 shows the right hemispheres of these monkeys, upon which the sites of the stimulating electrodes are marked. In the first diagram (*monkey 18*), the positions of all the electrodes implanted in that hemisphere are given, while in the remaining monkeys only the electrodes used in this study are shown. Even numbers indicate that the electrode was located on the right hemisphere, the odd numbers on the left. On the day following the operation, when the animals were awake and alert, they were placed in an examination chair and stimulated with 60 cycle sine wave current derived from the lighting circuit. The specific pairs of electrodes to be used as well as the required current strengths were decided after study of the responses elicitable from each electrode separately. This portion of the work has already been reported (1).

It is difficult to place an electrode on the anterior suppressor band accurately

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² These experiments were all performed in the Department of Anatomy, Vanderbilt University Medical School, while the senior author was on a short leave of absence.

from surface markings of the skull. Since, however, Bailey, *et al.* (5) found that the anterior suppressor band was closely related to area 8, the region giving head and eye movements, we chose for further study those electrodes eliciting such movements in a given animal from the several implanted in that general region. With such an electrode as one of a pair in each experiment, another electrode on the same side usually, which gave either arm or leg movements was used.

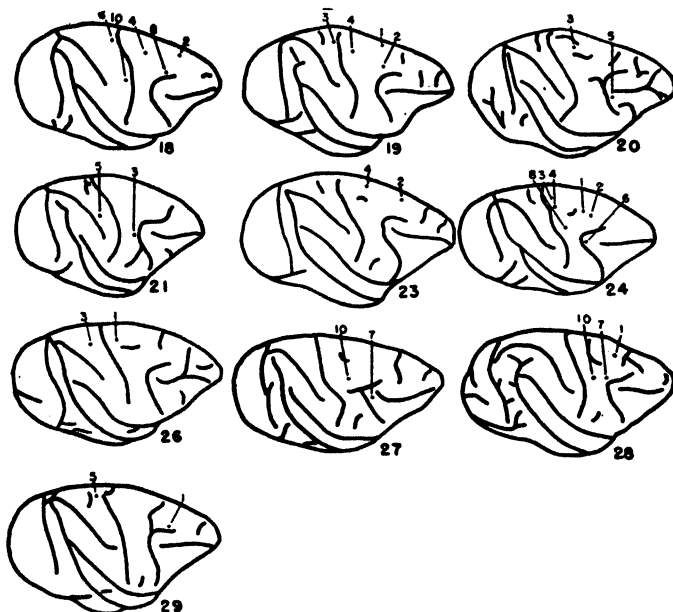


FIG. 1. DIAGRAMS SHOWING SITES OF IMPLANTED ELECTRODES

A condensed protocol giving pertinent data from one of these experiments (*monkey 23*) follows:

ELECTRODE	VOLTAGE	DURATION	RESPONSE
2	0.8	4 sec.	Head turned to left but no eye movements were observed.
4	0.8	4 sec.	With about $\frac{1}{2}$ second latency the leg was flexed.
Both	0.8	4 sec.	With the stimulus the left hind leg was flexed and head began to turn to left. Leg did not relax for 20 seconds.

The results are summarized in table 1. Following the number of each animal are 4 columns indicating the location of the primary movement (as in eye, head, arm or leg) elicited from each of the particular electrodes selected for combined stimulation. In many cases movements in more than one of these locations occurred with the particular strength of current used. Thus in *monkey 26* stimulation by electrode 1 produced movements in head, arm and leg. The order of stimulation is indicated by the symbols S—simultaneous, C—consecutive (routinely in consecutive stimulation the first electrode activated was the one from which head or head and eye movements

could be elicited), CB—where both orders were used and B—both consecutive and concurrent stimulation. Any change in the response is indicated in the following column. It is noteworthy that all changes in duration, amplitude or in extent of movement were toward an augmented response and in no case did the reverse occur. Clonus occurred in many animals at the current strengths selected. In several of these clonus occurred with the combined stimulation but was not present in the

TABLE 1. SUMMARY OF RESULTS

MONKEY NO.	PRIMARY MOVEMENT PRELIMINARY STIMULATION				ORDER OF STIMULATION	CHANGES IN RESPONSE	CLONUS	
	Head	Eye	Arm	Leg			Preliminary stimulation	Combined stimulation
18			4	6	S	In	Yes	Yes
19			1	3	S	In	No? ¹	Yes
	x ²	x ²	2, 4	2, 4	S	In	Yes	Yes
20	5	5		3	S	None	Yes	Yes
21	3		3	5	S	In	No	Yes
23	2	2		4	S	In	No	Yes
24	2		2	4	B	In? ³	Yes	Yes
				1, 3 ⁴	S	In	No	No
	6		6, 8		C	In	No	No
26			1, 3		CB	In	No	Yes
27	7	7	10		C	In	Yes	Yes
28	1	1	10		C	None	Yes	Yes
	7		10		CB	None	Yes	Yes
29	1			5	B	In	No	? ⁵

¹ At intensity of stimulation used in combined stimulation clonus appeared inconstantly during preliminary stimulation.

² There were no head and eye movements during preliminary stimulation but they did appear as part of the response to combined stimulation. This is indicated by the x in those columns.

³ This increase in response may be doubtful but if any change can be considered it was in this direction.

⁴ The voltage used for electrode 1 was below threshold, nevertheless knee flexion (the primary movement from electrode 1) was present. The response from electrode 3 was primarily inversion and dorsiflexion of the foot.

⁵ There was a clonic jerk following the response to combined stimulation and 3 such jerks when there was a 4-second delay between the 2 stimuli. However, with consecutive stimuli (at a slightly lower voltage) there was no evidence of clonus.

earlier single stimulation at the same intensity. The opposite effect was not seen. It should be emphasized that there was never any indication of inhibition either of movement or of after discharge. In summary, the responses to combined stimulation of two points were augmented as compared with the responses obtained from separate stimulation in 11 of the 14 attempts.

Aside from the possibility that stimulation of a suppressor band might have produced unconsciousness in an unanesthetized patient (6, 7), there are two responses which study of the literature would lead one to anticipate eliciting under the conditions of our experiments. These are: 1) a holding in abeyance of a cortical after discharge and 2) suppression of a motor response. The latency of the first of these is

very short even in the anesthetized animal where electrical stimulation of a suppressor band is said to inhibit immediately the clonic after discharge so readily produced by supraliminal stimulation of the motor cortex. One could have reasonably expected that mild clonus at least would have been inhibited rather than facilitated in the nonanesthetized animal by suppressor band stimulation. Since McCulloch (3) has shown that the duration and latency of suppression of a motor response are functions of the depth of anesthesia, it could be expected that such responses would have a very short latency in the unanesthetized animal and that suppression of a motor response should therefore have been apparent under the conditions of our experiments.

Two explanations may be advanced for the results obtained. It is possible that suppression is an artifact that can be elicited only under definitely abnormal conditions. Some support for this view may be gleaned from the literature. For example, how can one interpret the statement of Gellhorn (8) that "suppression is most easily elicited 18 or more hours after the operation" (his animals were under continuous Dial anesthesia) as other than that abnormal conditions must prevail in order to demonstrate this phenomenon. Furthermore, Clark, *et al.* (9) report that they were able to elicit suppression in dogs anesthetized with Dial but were unable to do so in the same animals when they were awake and alert. Since these workers used implanted electrodes, constant points were stimulated. When the present report of facilitation rather than suppression in the unanesthetized monkey is added to this, a rather strong case can be made. On the other hand, we are inclined to the view that both facilitation and suppression (4) can be elicited from the same cortical area, and that separation of these responses is possible under proper conditions. Under Dial anesthesia facilitative responses may be selectively eliminated as was shown in the dog by Clark, *et al.* (9). With other types of anesthesia suppression may be demonstrated with difficulty while in the unanesthetized animal facilitative responses may completely mask suppression. Such a statement, however, gives no clue concerning the rôle, *if any*, which suppression plays in the intact animals.

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EFFECT OF CARBON DIOXIDE ON BRAIN GLUCOSE, LACTATE, PYRUVATE AND PHOSPHATES^{1,2}

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CENTRAL nervous activity is considerably affected by procedures that cause changes within the carbon dioxide system of blood and tissue. For example, respiration of suitable concentrations of carbon dioxide induces hyperpnea, convulsions and anesthesia. Hyperventilation tends to induce relatively high-voltage, low-frequency cortical potentials and, in petit mal, typical seizure discharges (1). Conversely, petit mal seizures can be interrupted by increasing the carbon dioxide concentration of the inspired air (2). The total carbon dioxide content of arterial and jugular blood fluctuates abnormally prior to the onset of seizures (3). Repeated breathing of high concentrations of carbon dioxide appears to relieve certain neuroses (4). Moreover, breathing gas mixtures containing large (15-30%) amounts of carbon dioxide raises the convulsive thresholds to certain drugs and to electric shock, whereas it lowers the convulsive threshold to other drugs (5, 6).

At present there is insufficient information to warrant extensive consideration of the basic cellular mechanisms whereby the carbon dioxide system might affect central nervous activity. It appears that, due to relatively rapid rates of diffusion, the carbon dioxide and carbonic acid concentrations of the blood are the primary determinants of the hydrogen ion concentration of muscle (7). Generalization from this supports the impression that central control of respiration is more responsive to blood levels of carbon dioxide and carbonic acid than to the level of hydrogen ions (8). The central presence and localization of carbonic anhydrase (9) and the pathological variations in its concentration (10) suggest a special rôle for the carbon dioxide system in central nervous metabolism. The increase in cerebral blood flow (11) and consequent increase in oxygen tension of cortex (12) brought about by breathing increased concentrations of carbon dioxide and the decrease in flow (11) and oxygen tension (12) induced by hyperventilation are variables pertinent to considerations of central effects of the carbon dioxide system.

In the present work, in order to gain information that might further rational consideration of the rôle of carbon dioxide in central nervous metabolism, the cerebral hemispheres of cats were assayed for glucose, lactate, phosphates and pyruvate following changes which were effected in several ways within the carbon dioxide system.

EXPERIMENTAL

Procedures. Cats were employed as the experimental animals. They were paralyzed with dihydro- β -erythroidine, maintained with artificial respiration, and the

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skulls exposed for eventual freezing of the brain. One hour after onset of paralysis the animals were subjected to particular experimental procedures at the end of which the brains were frozen by pouring liquid air on the exposed skulls. Arterial blood was taken in heparin at appropriate times. The electrical activity of heart and brain was recorded during the experiments with a six-channel Grass Electroencephalograph. The EEG leads were taken from screw type electrodes placed bilaterally over the center of the cerebral cortex. Blood gases were measured manometrically with the usual Van Slyke apparatus. The hydrogen ion concentration of blood was measured with the Beckman *pH* meter, model G, using Model 290J electrodes to prevent escape of carbon dioxide. The preparation of the animals and other methods of assay have been reported in detail (13, 14).

Lactate and Pyruvate. The effects of various experimental conditions upon the concentrations of brain lactate and pyruvate are indicated in table 1. The highest level of lactate was found in the animals having the lowest level of blood carbon dioxide (*exper. 1*) and the lowest level in those breathing carbon dioxide plus 20 or more per cent oxygen (*exper. 8-14*). Intermediate levels were found in animals not given carbon dioxide, but having blood carbon dioxide contents higher than in *experiment 1* (*exper. 2-6*). The pyruvate levels in the animals given bicarbonate, carbon dioxide or both were lower than in those given no special treatment (compare *exper. 1-4* with 5-15).

The effect of breathing carbon dioxide on the concentrations of brain lactate and pyruvate was evident with 5 per cent (*exper. 8*), although not consistently (*exper. 7*), and was somewhat greater with higher concentrations. Administration during two minutes was sufficient to induce the lower levels of lactate and pyruvate (*exper. 10*).

The concentration of brain lactate bears some inverse relation to blood carbon dioxide levels (*exper. 1-5*); however, comparison of the lactate concentrations obtained when bicarbonate was administered (*exper. 5, 6*) with the concentrations found when carbon dioxide was given (*exper. 8-14*) indicates an exception to this apparent relation. The brain lactate level obtained when bicarbonate was administered alone (*exper. 5*) was higher than when carbon dioxide was also given (*exper. 6*). This, and the finding that administration of carbon dioxide alone resulted in the lowest lactate levels, suggests an inverse relation between the hydrogen ion concentration of blood and the concentration of brain lactate. However, the hydrogen ion concentration of blood cannot be the only determinant of brain lactate since the relatively high concentration of hydrogen ions in blood produced by injection of hydrochloric acid (*exper. 18*) was not accompanied by a particularly low level of brain lactate.

The concentration of blood oxygen may be concerned in the determination of the lactate level, since reduction of the oxygen content of the gas breathed to 10 per cent produced an elevation of lactate concentration (compare *exper. 9* and 15). Breathing 10 per cent oxygen alone presumably would not affect the concentration of brain lactate (14).

Carbon dioxide in relatively high concentration did not prevent the increase in brain lactate expected to accompany a suitable degree of hypoxia (*exper. 16, 17*).

TABLE 1. EFFECT OF CO₂ ON BRAIN LACTATE AND PYRUVATE¹

EXPER. NO.	CONDITIONS	BRAIN		FINAL BLOOD		
		Lactate	Pyruvate	CO ₂	pH	O ₂ Sat.
1	Room air	2.8 ± 0.2 (2)	0.22 ± 0.09 (2)	30 ± 3 (2)	7.65 ± 0.20 (2)	0.97 ± 0.02 (2)
2	Room air	2.2 ± 0.5 (13)	0.22 ± 0.11 (12)	32 ± 3 (3)		0.98 ± 0.03 (3)
3	Room air	1.4 ± 0.3 (6)	0.20 ± 0.08 (4)	47 ± 4 (4)		0.86 ± 0.07 (4)
4	100% O ₂ ; 10 min.	1.7 ± 0.3 (4)	0.21 ± 0.06 (4)	36 ± 6 (4)	7.33 ± 0.12 (4)	0.99 ± 0.03 (4)
5	I.v. bicarb.; room air	1.4 ± 0.2 (3)	0.12 ± 0.05 (2)	148 ± 50 (3)	7.84 ± 0.17 (3)	0.96 ± 0.03 (3)
6	I.v. bicarb.; 15% CO ₂ , 85% O ₂ ; 10 min.	1.2 ± 0.1 (2)	0.11 ± 0.01 (2)	158 ± 46 (2)	7.42 ± 0.02 (2)	1.08 ± 0.03 (2)
7	5% CO ₂ , 95% O ₂ ; 5 min.	1.8	0.19	44	7.07	0.98
8	Same as 7	0.9	0.13	46	7.29	1.00
9	15% CO ₂ , 85% O ₂ ; 10 min.	0.6	0.12	70		1.00
10	20% CO ₂ , 80% O ₂ ; 2 min.	0.8	0.07	61	6.99	1.00
11	20% CO ₂ , 80% O ₂ ; 5 min.	0.7 ± 0.1 (3)	0.07 ± 0.01 (3)	58 ± 5 (3)	6.94 ± 0.03 (3)	1.00
12	20% CO ₂ , 80% O ₂ ; 10 min.	0.4	0.06	56	6.92	1.00
13	20% CO ₂ , 80% O ₂ ; 20 min.	0.8	0.06	62	6.95	0.95
14	30% CO ₂ , 70% O ₂ ; 10 min.	0.6	0.09	60		1.00
15	15% CO ₂ , 10% O ₂ , 75% N ₂ ; 10 min.	1.4 ± 0.05 (2)	0.11 ± 0.01 (2)	57 ± 7 (2)		0.37 ± 0.08 (2)
16	15% CO ₂ , 6% O ₂ , 79% N ₂ ; 10 min.	9.6	0.26	57		0.25
17	100% CO ₂	8.32		106	6.50	0.04
18	I.v. HCl; 7 min.	1.5		32	7.10	0.69
19 ²	15% CO ₂ , 85% O ₂ ; 10 min.	1.7 ± 0.1 (3)	0.14 ± 0.03 (3)			
20	15% CO ₂ , 85% O ₂ ; cyanide i.v.	5.6		38		1.02
21	Convulsions; room air	6.2	0.22 ± 0.11			
22	Convulsions; room air	5.0 ± 0.7 (2)	0.17 ± 0.07 (2)			
23	Convulsions 15% CO ₂ , 85% O ₂	2.2 ± 0.6 (5)	0.18 ± 0.09 (3)	60 ± 4 (5)	7.01 ± 0.06 (3)	1.02 ± 0.050 (5)
24	Convulsions 15% CO ₂ , 10% O ₂ , 75% N ₂	2.5 ± 0.1 (2)	0.10 ± 0.02 (2)			

¹ Where appropriate, 3 figures representing the mean, standard deviation and no. of animals (in parentheses) are given for each assay in a particular experiment. Concentrations of lactate and pyruvate are expressed in mm/kg. of tissue, and CO₂ content in volumes %. O₂ saturation signifies the ratio of the O₂ content of the blood as drawn to that after equilibration with room air. The data for lactate and pyruvate in *exper. 2* and *21* have been reported previously (13). *Exper. 1-3* differ in rates of ventilation used. In *exper. 5* and *6*, 60 ml. of 0.5 M sodium bicarbonate was injected intravenously during the period of time indicated. In *exper. 6* administration of CO₂ was begun immediately after initiation of bicarbonate injection. In *exper. 18*, 80 ml. of 0.05 M HCl was injected intravenously during the period indicated. In *exper. 20*, 0.8 mg. sodium cyanide/kg. body weight was injected immediately after the animal began breathing CO₂. Convulsions were produced by intravenous injection of about 15 mg. metrazol/kg. body weight. Brains frozen at end of 180 seconds of convulsive activity essentially like that recorded in (13).

² Carotids ligated immediately after the animals began breathing CO₂ mixture.

and 20). The concentration of lactate found when the carotids were ligated during administration of carbon dioxide (*exper. 19*) was higher than when carbon dioxide was given alone. Therefore, it appears that cerebral circulation is involved in the effect of carbon dioxide on the concentration of lactate. It should be pointed out here that in the cat cerebral function can be maintained by the vertebrals alone.

The foregoing remarks about lactate apply to brain pyruvate, although in lesser degree.

Summarizing, it appears that the concentrations of brain lactate and pyruvate bear an inverse relation to the levels of blood carbon dioxide, hydrogen ions, oxygen and cerebral blood flow.

TABLE 2. EFFECT OF CO₂ ON BRAIN PHOSPHATES AND GLUCOSE¹

EXPER. NO.	CONDITIONS	BRAIN					BRAIN/PLASMA GLUCOSE
		IP	PC	ATP	ADP	Glucose	
1	Room air	4.7± 1.8 (14)	2.2± 0.5 (14)	1.3± 0.5 (10)	1.1± 0.7 (10)	4.2± 1.1 (11)	0.36± 0.08 (11)
2	Room air	4.2± 1.3 (7)	2.6± 0.6 (9)	1.5± 0.6 (7)	0.9± 0.5 (7)	3.9± 1.2 (10)	0.38± 0.02 (10)
3	Bicarbonate	3.9± 0.9 (5)	3.0± 0.5 (5)	1.7± 0.2 (5)	0.7± 0.1 (5)	3.8± 0.6 (5)	0.43± 0.07 (5)
4	CO ₂	4.3± 0.6 (5)	2.2± 0.1 (7)	1.4± 0.1 (7)	0.9± 0.1 (7)	4.9± 1.9 (10)	0.33± 0.05 (10)
5	Convulsions; room air	5.8	1.7	0.6	2.1	1.7	0.21
6	Convulsions; room air	6.0± 0.7 (2)	0.8± 0.3 (2)	0.9± 0.2 (2)	1.9± 0.6 (2)		
7	Convulsions; 15% CO ₂ , 85% O ₂	3.6± 0.5 (4)	2.1± 0.2 (4)	1.4± 0.2 (4)	0.7± 0.2 (4)	5.5± 2.0 (5)	0.26± 0.07 (5)

¹ Concentrations are expressed in mM/kg. Portions of the data for *exper. 1* and *5* have been reported previously (13). The animals used in *exper. 2* were those of *exper. 1, 3, and 4*, table 1; in *exper. 3* were those of *exper. 5 and 6*, table 1; in *exper. 4* were those of *exper. 8-14*, table 1. The animals used in *exper. 7* were those of *exper. 24*, table 1.

Acid-soluble Phosphates and Glucose. The concentration of brain phosphates, glucose and ratio of brain to plasma glucose under various experimental conditions are given in table 2. The results for *experiments 2-4* were derived from data obtained in relatively similar individual experiments that differed in certain details. Comparison between the individual experiments was made but revealed no notable differences.

The data indicate that neither intravenous injection of considerable amounts of bicarbonate nor respiration of relatively high concentrations of carbon dioxide had any appreciable effect on the concentrations in brain of inorganic phosphate, phosphocreatine, adenosine phosphates or glucose.

Convulsions. Data indicating the effect of breathing carbon dioxide on the changes in brain accompanying seizures are given in tables 1 and 2. As shown by comparison of *experiments 2* with *19 and 20*, table 1 and other earlier work (13, 14, 16), convulsions are accompanied by an increase in brain lactate and by decreases

in the concentration of brain high-energy phosphates and glucose. (Compare *exper. 1* with 5 and 6, table 2.)

Comparison of *experiments 9* and 23, table 1, shows that seizures occurring during administration of carbon dioxide were also accompanied by an increase in brain lactate. The percentage increase was about the same as found in air; however, the actual increase was considerably less. Further, the increase in lactate accompanying convulsions in animals breathing carbon dioxide and 10 per cent oxygen was less (compare *exper. 15* and 22) than in animals breathing room air.

Comparison of *experiments 4* and 7, table 2, indicates that seizures occurring during administration of carbon dioxide, in contrast to the results in room air (compare *exper. 1* and 2 with 5 and 6), were not accompanied by appreciable change in the concentrations of high-energy phosphates.

Although the concentration of brain glucose in the convulsed animals breathing carbon dioxide was higher than in the controls (compare *exper. 4* with 7), the ratio of brain to plasma glucose was lower. Since, in the absence of rapid change in rate of metabolism or rate of supply of glucose, the ratio of brain to plasma glucose is relatively constant (*exper. 1-4*) (17), this decrease in ratio may represent a decrease in concentration of brain glucose effected by the seizures.

Summarizing, breathing carbon dioxide practically prevents the decrease in high-energy phosphates accompanying seizures, limits the increase in lactate, and has little effect on the decrease in brain to plasma glucose ratios.

DISCUSSION

It is known that breathing carbon dioxide increases the rate of cerebral blood flow and that hyperventilation decreases the rate of flow (11) and the change in cerebral oxygen tension is in the expected direction (12). The rates of blood flow during administration of carbon dioxide, in the control state, and during hyperventilation are in arbitrary units about 4, 2, and 1 respectively (11). The concentrations of brain lactate in what roughly corresponded to those several states were about 0.7, 1.4, and 2.8 mm per 1000 grams respectively (compare *exper. 8-14*, 3 and 1, table 1). This nice inverse correspondence between probable rates of blood flow and concentrations of brain lactate, admittedly fortuitous, and the observation that ligation of the carotids limits the fall in lactate level accompanying administration of carbon dioxide suggest a major rôle for blood flow in determining the concentrations of lactate and, to a lesser extent, pyruvate in brain. Thus, it may be proposed that all of the present procedures that resulted in relatively low levels of brain lactate and pyruvate mediated their effect by producing a high rate of blood flow and, conversely, those resulting in high levels (except seizures) did so by effecting a relatively low rate of flow.

Blood flow might act by changing the effective concentrations of these substances in blood, e.g., an increase in rate of blood flow would be comparable to decreases in concentration of the substances in blood. From this, some relation between the concentrations in blood and brain would be expected. However, no consistent relation between blood and brain concentrations was found, and the available evidence indicates that markedly high levels of blood lactate have little, if any, influence on

the concentration in brain (18). The same low levels of brain lactate and pyruvate, induced by administration of carbon dioxide, were found in the presence of two-fold differences in blood levels. Animals breathing air and having high levels of brain lactate and pyruvate had blood levels lower than in animals breathing carbon dioxide and having, consequently, low concentrations of these substances in brain. Thus, the essentially mechanical aspects of blood flow hardly suffice as explanation of its relation to brain lactate and pyruvate.

The oxygen tension of cerebral cortex varies directly with blood flow (11). The finding that decrease in the concentration of oxygen in the carbon dioxide mixture breathed to 10 per cent limited the fall in lactate and pyruvate levels induced by the carbon dioxide suggests that the oxygen tension of brain may inversely affect the brain levels of the substances in question. In the case of lactate such an effect might proceed by varying the concentration of reduced diphosphopyridine nucleotide available for reduction of pyruvate, e.g., high oxygen tension would decrease the concentration of reduced nucleotide and consequently the rate of formation of lactate from pyruvate.

In addition, the level of pyruvate available for reduction might be reduced by increased efficiency of the Krebs cycle at increased oxygen tensions. There is a suggestion of this in the data since pyruvate drops with carbon dioxide and oxygen mixtures. It does not drop with 100 per cent oxygen but there is only a small increase in oxygen tension of the cortex under these conditions compared to carbon dioxide plus oxygen (6).

There is another related rationale for the observed changes in lactate under carbon dioxide inhalation. Brain is reported to have an unusually high rate of aerobic glycolysis (19). It has been shown in rat liver that one in eight carbons of newly formed glycogen comes from bicarbonate (20) and it was proposed that the glycogen was formed from carbon dioxide and phosphopyruvic acid. Moreover, it is known that carbon dioxide can be assimilated (21, 22). Presumably these reactions can also occur in brain since it has been demonstrated that carbon dioxide is incorporated into citrate in brain *in vivo* (23). All of these reactions would have the effect of limiting the amount of pyruvate available to accept hydrogen from the glycolytic mechanisms and might result in lowered lactates.

Craig (24) has reported that increase in hydrogen ion concentration and total carbon dioxide (from 1 per cent to 5 per cent) results in an increase in the rate of aerobic glycolysis in brain slices *in vitro*. Levels of carbon dioxide above 5 per cent had no further effect. The present work is not in obvious agreement with this report, but it should be noted that the level of carbon dioxide in the residual alveolar air is approximately 5 per cent (25). The exact carbon dioxide tension of the cortex in these artificially ventilated animals is not known although the value of 30 volumes per cent in the arterial blood is suggestive of values considerably above 1 per cent. It would appear that in the *in vitro* studies (24) changes were noted at values below normal carbon dioxide levels but not above.

There is little doubt that carbon dioxide has profound effects upon the levels of lactate, pyruvate and phosphates in the brain. The mechanism by which these effects are mediated remains obscure. The most probable explanation is that the

resulting increase in blood flow and oxygen tension enabled the brain to more nearly balance energy demands with oxidative processes and as a consequence less lactate was formed and high energy phosphate reservoirs were not depleted. The effects are especially marked during convulsions where energy demands are high. The other possibilities discussed above may contribute to the overall effect, but it is the authors' feeling that theirs is a minor rôle.

SUMMARY

The brains of normal and convulsing cats subjected to conditions designed to alter the carbon dioxide—bicarbonate—carbonic acid system were analyzed after freezing with liquid air. In normal animals, breathing 10 to 30 per cent carbon dioxide mixtures resulted in a lowering of lactate and pyruvate levels and had little effect on glucose or phosphate concentrations. In convulsed animals these conditions markedly limited the expected rise in lactate, pyruvate and inorganic phosphate and prevented the fall in high-energy phosphates. There was a rise in brain glucose but little effect was found on brain/plasma glucose ratios. The results are discussed with relation to possible mechanisms and the effects of carbon dioxide on the central nervous system.

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INFLUENCE OF INCREASED TEMPERATURE ON ACTIVITY OF THE CEREBRAL CORTEX¹

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THE purpose of the present investigation is to determine the upper limits of brain temperature compatible with the preservation of cortical activity and the effect of increased temperature on the electrocorticogram as well as on the excitability of the motor cortex to electrical stimuli. The effect of temperature on the cortex is known for a small range only from the experiments of Hoagland (1), who studied in man the effect of fever treatment on the EEG and found that between 38.5 and 40° C. the frequency of the alpha potentials increased with increasing temperature. Clinical observations of the highest temperature compatible with life are of little value for the present problem since death may be caused by the action of high temperature on organs other than the brain. A more direct approach to the problem of relation between brain activity and temperature is found in the work of Heymans and Heymans (2) who in experiments on the isolated head of a dog noted cessation of respiratory movements at 45.4° C. Unfortunately the temperature was not recorded in the brain itself but through a sublingually placed thermometer which does not accurately indicate the temperature of the brain (3). Experiments on spinal reflexes showed an increase in response as the rectal temperature rose to 42° but some damage occurred since on cooling the reflex was smaller than in control conditions (4).

METHODS

In 16 cats injected with 0.45 cc. per kilo dial urethane (i.p.) the cortex was exposed widely and the potentials were recorded through bi-polar silver electrodes with an Offner inkwriter. Furthermore the motor cortex was stimulated with condenser discharges (Goodwin stimulator) and the effect was recorded by means of EMGs as previously described (5). Cortical temperatures were

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recorded with iron-constantan thermocouple junctions placed in the ends of long, 22-gauge needles and introduced into the cortex approximately 1 mm. below the pia mater between the tips of the recording bi-polar electrodes. The latter placement in no way interfered with either potential recording or with application of electrical stimuli in the studies of motor cortex reactivity and insured direct relation between the cortical area studied and the recorded temperature. Thermocouples placed directly into the cortex without the support of a metal needle showed approximately the same temperature readings of the heated brain as those placed inside the needles indicating that thermal conduction of the latter did not significantly influence the temperature reading.

The cortex was heated by direct radiation from a 250-watt incandescent infra-red lamp placed at varying distances from the cortical surface according to the temperature desired. The remainder of the animal and especially the remaining cortex were shielded from the radiation by suitably placed strips of asbestos and layers of paper toweling, and from significant temperature elevations by a stream of cool air; the latter was primarily directed over the covered cortical areas which were still to be used in the experiment.

Degree and rate of heating were varied as follows: 1) Heating of cortex from 35 to 50° C. in 48 min. ('slow heating'). 2) Heating of cortex from 35 to 50° C. in 12 min. ('fast heating'). 3) Heating of cortex for 30 minutes at a given temperature. 4) Periodic heating of cortex to given temperature, each heating lasting 3-8 minutes with a total heating time of 30 minutes.

Evaporation from the cortex was minimized by the application of mineral oil. Rectal temperature was recorded also.

RESULTS

The spontaneous electrocorticogram decreased progressively in amplitude as the temperature of the cortex was elevated, and this effect occurred with both 'slow' and 'fast' heating (fig. 1). In general it was found that the amplitude declined at a somewhat higher temperature under the influence of fast heating than under that of slow heating.

Heating to 50° C. regularly produced a decline in the amplitude of the potentials to 30-50 per cent of the control, as well as an excitation which was indicated by the disappearance of dial potentials. After the temperature had been lowered to the original level close inspection showed no fundamental changes in frequency of potentials as a result of the intervening heating procedure, irrespective of the rate of temperature elevation (fig. 2).

The reactivity of the motor cortex to electrical stimulation (condenser discharges) as indicated by electromyograms usually remained at a relatively constant level until a temperature of about 45° to 47° C. was reached. A small further temperature elevation usually resulted in a relatively rapid rise in threshold (disappearance of the muscular response to cortical stimulation) at a time when the electrocorticogram showed no corresponding decrease in amplitude (fig. 3).

The temperature at which this rapid change in threshold occurred varied in 5 experiments with 'slow heating' between 45.5° and 49° C. (mean 46.5°) whereas in a series of 4 experiments with 'fast heating' this change occurred at a mean temperature of 50.9° C.

Table 1 shows the 'critical' temperature at which a change in motor cortex threshold was observed and also the threshold of the motor cortex on recovery. It is notable that in 2 out of 5 experiments the cortex could be heated to 50° without a significant change in cortical threshold. If as the result of heating a distinct increase in the threshold of the motor cortex occurred, it was noted that slight variations in the intensity of cortical stimulation failed to alter the amplitude of the EMG

whereas in the normal cortex an increase in cortical stimulation is followed by an increase in the amplitude of the EMG (5, 6).

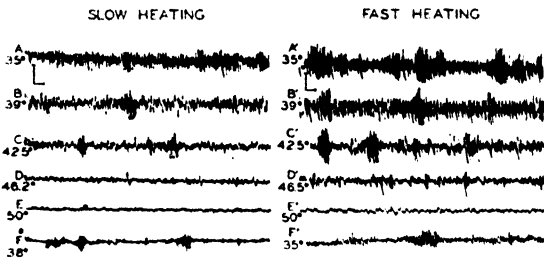


Fig. 1. EFFECTS OF INCREASED TEMPERATURE on the electrocortigram and comparison of the effects of 'slow' and 'fast' heating in the same experimental animal. A and A', control. B-F, 'slow' heating, 35°-50° C. in 48 min. and recovery. B'-F', 35°-50° C. in 12 min. and recovery. Calibration in all tracings: 1 sec. and 300 μ v.

Fig. 2. EFFECT OF HEATING on wave form and frequency of ECG. 1, control, 35° prior to heating. 2, record at 35° after 'slow' and 'fast' heating to 50° C.

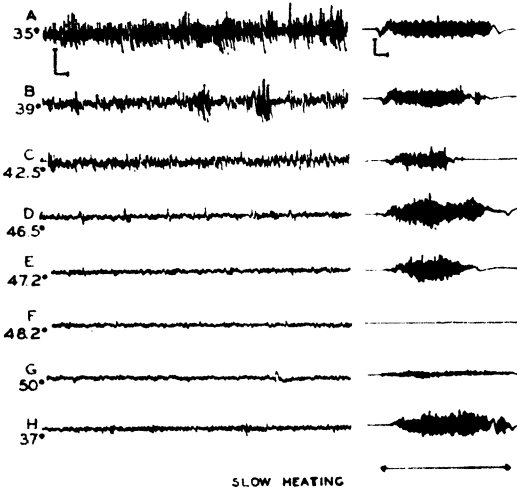
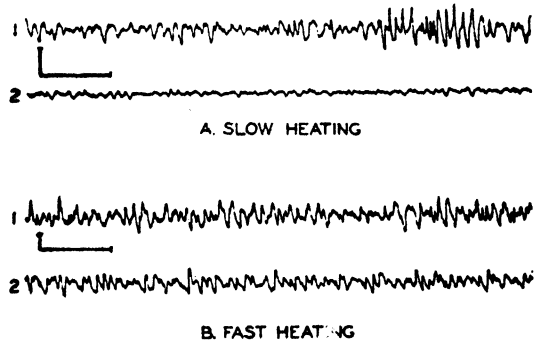


Fig. 3. EFFECT OF 'SLOW' HEATING on reactivity of the motor cortex and the ECG. A, control, ECG and EMG of the tibialis anterior muscle. (Stimulation of the motor cortex at 3.3 v. and 83/sec.) B-G, effect of heating to 50° C. H, recovery.

Exposure of the cortex for 30 minutes to a given temperature which varied in different experiments between 44 and 47° C. abolished dial potentials in most instances during heating and caused a reduction in background activity in all but one experiment. On return to the control temperature dial potentials returned in 5 out of 9 experiments. Although there was some reduction in the amplitude of the background activity in most of the experiments the frequency of discharge was similar to that seen in the unheated control experiments. Heating of the cortex to 44 or 46°

C. for 30 minutes did not appreciably alter the response of the motor cortex to condenser discharges whereas heating to 47° C invariably raised the threshold. When the cortex was subjected to periodic heating at a given temperature (*method 4*, see above) the results were similar in that threshold changes were minimal at 46° but considerable at 47° C.

TABLE 1. INFLUENCE OF 'SLOW' HEATING TO 50° C. ON REACTIVITY OF MOTOR CORTX INDICATED BY ELECTROMYOGRAMS

SLOW HEATING				FAST HEATING			
Exper.	Threshold	Critical ¹ Temp.	Threshold Recovery Period	Exper.	Threshold	Critical ¹ Temp.	Threshold Recovery Period
	<i>Volts</i>	<i>°C.</i>	<i>Volts</i>		<i>Volts</i>	<i>°C.</i>	<i>Volts</i>
25	4.8	45.5	6.3	25	3.3	46.5	6.3
26	4.8	48.0	6.3-8.0	26	3.3	52.2	9.5
28	3.3	47.25	3.3	27	6.3	55.0	9.5
30	4.8	49.0	4.8	28	4.8	50.0	8.0
31	2.3	45.5	6.3-8.0				
Approx. Mean.....		46.5		Approx. Mean.....		50.9	

¹ Temperature at which the threshold stimulus is no longer effective.

TABLE 2. VERTICAL GRADIENT OF CORTICAL TEMPERATURE ON HEATING

PROCEDURE	CORTICAL POINT	TEMP. IN °C. AT DISTANCE OF THERMOCPL. JUNCTION BELOW PIA MATER IN MM.								RECTAL TEMP.
		0	1	2	3	5	10	1 ¹	0 ²	
Prior to heating	A	32.5	33.5	34	34.5	35	35.7	33	32	38
	B	33.2	33.5	33.7	34	34.5	35.5	33.7	33.5	38
	C	31	32	32.5	33	33.2	34.5	31.2	30.5	38
Moderate heating	A	39.5	40	40	40	39.5	37.5	39.7	39.7	38
	B	38.2	38.5	38.5	38.5	38.2	37.5	38.5	38	38
	C	39.2	39.7	39.7	40	39.5	38.2	39.5	39.5	38
Intense heating	A	49.5	49	49	48.5	46	41	50	50	38
	B	44.5	44.5	44.5	44	43.7	42	45	45	38
	C	48.0	49.2	49.5	49.5	49.5	47.5	49.2	48	38.5

¹ Thermocouple junction visible at the cortical surface.

² Thermocouple junction returned to this level for check of temperature.

Finally temperature measurements at the surface and at different depths of the brain are presented in table 2. Without application of specific heating devices the temperature of the exposed cortex varies between 31 and 33° C. while rectal temperature is normal. Excitability (responses to stimulation of the motor cortex) and cortical potentials remain very good for many hours under these conditions. On heating as used in this study the difference in temperature between the cortical surface and the first 2 mm. below the surface is minimal, suggesting that the temperature of various cortical layers is not significantly different from that recorded at a depth of about 1 mm. which was the standard procedure in this study. The table shows also that heating the brain even to 50° C. did not alter the rectal temperature.

COMMENT

The experiments show a rather high resistance of cortical activity to heating since temperatures up to 46° C. are tolerated for 30 minutes with little change in cortical motor threshold and with only slight decline in amplitude of the potentials. The disappearance of dial potentials on heating is interpreted as a sign of excitation, while the fact that on recovery from heating the amplitude of the potentials is reduced without any change in the frequency pattern suggests that heating interferes with the number of discharging neurons but not with their degrees of synchrony. It was also observed that whereas prior to heating a slight increase in intensity or frequency of cortical stimulation resulted in a notable increase in the amplitude of the EMG, this was no longer the case after heating. Since previous work (5-7) had shown that the amplitude of the EMG is an indicator of the number of cortical neurons which had been activated by stimulation, the increase in threshold to electrical stimulation after heating and the failure of the amplitude of the EMG to increase with further increase in frequency or intensity of cortical stimulation suggest that the low threshold neurons are particularly sensitive to elevated temperature. The damage resulting from exposure of the cortex to temperatures higher than 46° C. is not irreversible as seen in experiments in which the cortex was heated to 50° or slightly more. The reduction in amplitude of the action potentials noted after return to the control temperature increases with increasing temperature. It was found also that a considerable degree of electrical cortical activity may persist after temporary heating of the cortex produces permanent abolishment of the motor cortex reactivity to electrical stimuli.

SUMMARY

The exposed cortex of anesthetized cats was heated with an infra-red lamp and the temperature of the cortex, its action potentials and the reactivity of the motor cortex to electrical stimulation (indicated by the amplitude of the EMG of one or several muscles) were recorded. It was found that with increased temperature (above 45° C.) dial potentials disappear and are replaced by smaller and more frequent potentials (excitation). On cooling, the original pattern of the ECG is restored. On further heating the amplitude of the potentials decreases in proportion to the degree and duration of heating. Heating to 46° C for 30 minutes may be tolerated without change in threshold of the motor cortex. Even heating to 50° does not abolish cortical activity but raises the threshold of the motor cortex to electrical stimulation and leads to a partial loss in electrical activity as indicated by a decrease in amplitude of the ECG. It is suggested that the neurons which are relatively thermosensitive are those showing a low threshold to electrical stimulation.

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CHEMICAL FACTORS INFLUENCING MUSCULAR ATROPHY

ERNST G. HUF AND ERNST FISCHER

With the technical assistance of CAROLYN R. WEATHERFORD AND LUKE R. RADER

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SKELETAL muscles atrophying as a result of denervation undergo physico-chemical changes as well as some remarkable biochemical changes. The most important ones are: a wasting of substance, of protein in particular, takes place; the oxygen consumption of the denervated muscle is irregularly increased; the lactic acid formation is increased while glycogen and creatine content are decreased (1). Little attention has been called to the fact that these changes are the main biochemical characteristics of hyperthyroidism.

Muscle weakness in Graves disease shows features which can be compared with an acute muscular dystrophy (2-4). Ayer, Means and Lerman (5) have reported one case of a patient who rapidly developed a progressive, unilateral muscular atrophy with signs of thyrotoxicosis (BMR + 58%). After treatment with iodine and subtotal thyroidectomy, not only the exophthalmic goiter but the muscular atrophy as well disappeared. As early as 1898, Askanazy (6) made a study of 4 cases of hyperthyroidism in which he found a degenerative atrophy of the cells and the nuclei in striated muscles. More recently Feinstein, Pattle and Weddell (7) have reported that the fibrillation during denervation atrophy sets in earlier when rabbits are fed with thyroid; and that thyroidectomy retards the onset of fibrillation. These observations indicate that there is a relationship between muscular atrophy, thyroid function and iodine metabolism in the body. Additional data supporting this viewpoint are presented in the following study, in which three problems have been investigated: *a*) influence of thyroxine, thiourea and other drugs on muscular atrophy; *b*) influence of diets free of carbohydrate or fat or protein on muscular atrophy; and *c*) influence of the atrophying muscle on the weight of the thyroid gland.

METHODS

Animals. Adult male albino rats weighing 166 to 185 gm. at the beginning of the experiments were used in all experiments. Each batch of animals was subdivided into control and several experimental groups. The animals were kept in groups of 5 to 10 in metal cages of suitable dimensions with wire screen bottoms. Tap water and food (Purina dog chow checkers if not otherwise stated) were given daily *ad libitum*. The experiments were made during the months of February through December, 1947 and October through November, 1948. A small air conditioning unit in the animal

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room helped to reduce the greatest fluctuations in temperature, which was kept within a range of 20–28° C. The weights of the animals were recorded, as a rule, 2 to 3 times a week.

Denervation. Atrophy of the hind limb muscles was induced by aseptically resectioning the tibialis nerve on the left side, or in some rats resectioning the nerves on both sides.

Drugs and Daily Doses (per 100 gm. body weight, in 0.2 or 0.4 ml. of solvent). Thyroxine (Squibb crystals), 20 µg.; thiourea, C. P. (Eimer and Amend), 50 mg.; BAL (Hynson, Westcott and Dunning, Inc.), 4.7 mg.; iodoacetic acid (neutralized, Eastman Kodak Co.) 1.2 mg.; mapharsen (Parke, Davis Co.), 1 mg.; KI, 10 mg.; As₂O₃, 3 µg.; and 2,4-dinitrophenol, 1 mg. Thiourea and KI were given by stomach tube; the other drugs were injected subcutaneously. All drugs but BAL, which was dissolved in oil, were given in aqueous solution. The treatments lasted from 8 to 24 days and were started either on the day of operation or the following day.

Calculations. At the end of the experimental periods, the rats were killed either by a blow on the head or by ether inhalation. The gastrocnemius (+ m. soleus) of both sides, and, in the majority of cases, the thyroid glands, were taken out and kept separately in a small moist chamber for a few hours in the cold room until the tissues were weighed on an analytical balance.

In all the tables presented here, 'N' is the wet weight of the normal (control) gastrocnemius muscle in mg/100 gm. of body weight (b.w.); 'D' is the weight of the denervated gastrocnemius muscle in mg/100 gm. b.w.; 'd' is the weight of the denervated muscle expressed in percentage of the control muscle; 'a' is the degree of muscular atrophy in per cent ($100-d = 100 N-D/N$). Standard errors, significance (*S*) and *P*-values have been calculated for all 'd' values according to Burn (8). As can be seen from table 1, in normal rats and in rats with both hind limbs denervated the left and right gastrocnemius muscle differed in weight by about ± 1 per cent.

RESULTS

Effect of Thyroxine, Thiourea and Other Drugs on Muscular Atrophy. Average values for muscular atrophy at the end of 16 days (table 1, column 10) were found to be: 55.1 per cent for untreated rats; 56.6 per cent for rats injected with mammalian Ringer ($P > 0.2$); 61.8 per cent for rats daily injected with thyroxine (P^* and $P^+ < 0.01$); 51.9 per cent for rats daily fed with thiourea (P^* and $P^+ < 0.01$); and 52.5 per cent for rats daily fed with KI ($P^* = 0.03$; $P^+ = 0.01$). The *P*-values permit one to conclude that thyroxine accelerates while thiourea and KI inhibit muscular atrophy. Corresponding results on the effect of thyroxine and thiourea on muscular atrophy were obtained from a group of 70 rats, subdivided into smaller groups with denervation ranging from 9 to 24 days.

Similar experiments have been carried out in which the rats were given daily, over a period of 14 to 15 days, 2,4-dinitrophenol (DNP; 28 rats), arsenic (26 rats), mapharsen (5 rats), BAL (10 rats) and iodoacetic acid (6 rats). No effect was seen with mapharsen, BAL and iodoacetic acid. The effect on DNP and arsenic on muscular atrophy is given in figure 1. The lower curve shows the rate of muscular atrophy in a group of 68 control rats with denervation ranging from 9 to 21 days. The upper

curve refers to the treated rats. To a first approximation, the curves may be regarded as straight lines. Therefore, after calculating the degree of muscular atrophy per day, all the experimental data can be combined for statistical analysis. The *P*-values are <0.01 indicating that DNP and arsenic accelerated muscular atrophy.

TABLE 1. DEPENDENCE OF MUSCULAR ATROPHY ON TREATMENT WITH THYROXINE, THIOUREA AND POTASSIUM IODIDE

1	2	3	4	5	6		7	8	9	10	11
EXPER.	TREATMENT	DENERVATION	NO. RATS	CHANGE IN BODY WT.	AV. WT. OF GASTROCNEMIUS		MUSC. ATROPHY (a)	SIGNIFICANCE (S)	THYROID GLAND	SIGNIFICANCE (S)	
					Normal (N)	Denerv. (D)					
				%	mg/100 gm. b.w.						%
1	None	None	16		569.6±5.56(r) 568.1±6.47(l)					6.17±0.278	
2	None	Both sides	13	+23.3		272.3±7.37(r) 276.1±6.78(l)				5.83±0.199	1.0
3	None	One side	24	+37.5	578.3±7.31	260.0±4.21	55.0			5.38±0.193	2.3
4			8	+26.4	577.3±15.80	257.8±12.20	55.3				
3+4			32	+35.0	578.0±6.61	259.4±4.27	55.1				
5	Ringer	One side	16	+36.4	600.7±10.00	260.4±6.71	56.6	1.1*			
6	Thyroxine	One side	14	+13.1	587.1±10.10	227.1±4.89	61.3			4.30±0.240	5.1
7			7	+17.1	501.7±20.28	187.0±10.61	62.7				
6+7			21	+14.2	558.6±12.90	213.7±5.46	61.8	5.4* 3.5+			
8	Thiourea	One side	17	+12.4	572.6±7.70	275.6±6.64	51.9			13.30±0.401	14.6
9			12	+18.6	571.5±12.32	274.3±10.22	52.0				
8+9			29	+14.7	572.1±6.68	275.1±5.64	51.9	2.6* 3.2+			
10	KI	One side	8	+15.9	570.1±12.90	270.4±5.32	52.5	2.2* 2.8+		6.20±0.376	0.06

Denervation period and duration of treatment 16 days. Daily doses/100 gm. body weight: thyroxine 20 μ g.; thiourea 50 mg.; KI 10 mg. Experiments 4, 5, 7 and 9 in Oct.-Nov., 1948. All other experiments in March and April, 1947.

* and + S-values calculated in comparison with untreated and Ringer treated rats respectively.

Effects of Diets on Muscular Atrophy. White male rats, weighing initially 35 to 50 gm. were fed with crisco-sugar-casein diet *ad libitum*. The diet was supplemented by vitamins.² Tap water was used as drinking water. Five to six weeks later, when the

²Per 5000 gm. food: 2 methyl-naphthoquinone 25 mg.; thiamine HCl 25 mg.; pyridoxine HCl 25 mg.; riboflavin 50 mg.; Ca-pantothenate 250 mg.; p-aminobenzoic acid 50 mg.; nicotinic acid 100 mg.; inositol 2000 mg.; choline HCl 5000 mg.; Vit. D (10 USP U in propylenglycol Drisdol) and one drop of biotin concentrate (General Biochemicals) once a week; Vit. A (20 USP U = 127 μ carotene) in Wesson oil twice weekly.

rats had a body weight of 110 to 120 gm., the left hind limbs were denervated. The operated rats were subdivided into 4 groups. One group (the control group) was fed with the complete diet mentioned above (FCP-diet). The other groups were fed with diets lacking either fat (-CP) or carbohydrate (F-P) or protein (FC-). The composition of the diets is given in table 2. Daily measurements of the food intake were recorded and, from the physiological heat values of the diets, the caloric intake of the rats was calculated; this was almost the same in the different groups (1070 to 1180 Kcal/m² per 24 hrs.). The body weights of all the rats were determined at the beginning, in the middle, and at the end of the denervation experiments. The degree of muscular atrophy was determined as described earlier.

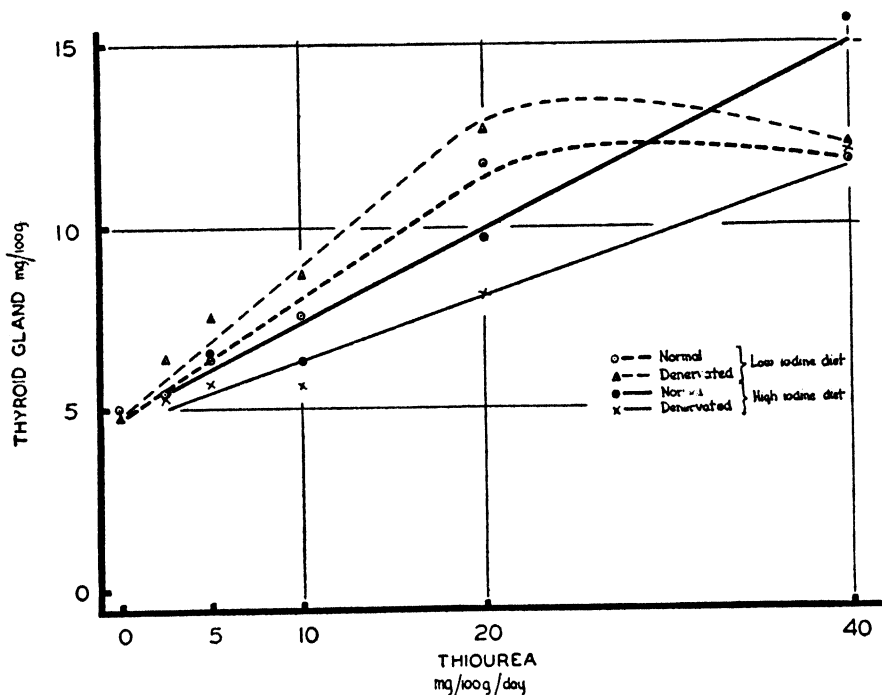


Fig. 1. DEPENDENCE OF RATE of muscular atrophy on duration of denervation. Normal rats and rats treated with DNP and arsenic.

Table 3 shows that a diet lacking either fat or carbohydrate did not change significantly the degree of muscular atrophy. Lack of protein, however, delayed the rate of muscular atrophy as can be seen by comparing in table 3, line 4 with lines 1 to 3. The difference in the degree of muscular atrophy in rats having protein (casein) in their food or lacking it is statistically significant.

Influence of Muscle Atrophy on Weight of the Thyroid Gland. Table 1, column 10 shows, as was expected, that rats which were treated with thyroxine (including those denervated on one hind limb) have considerably smaller thyroid glands ($P < 0.01$), while those treated with thiourea have an increased weight of the thyroid gland ($P < 0.01$). Treatment with KI had apparently no influence on the average weight of the thyroid gland. It was noted, however, that non-treated rats, denervated on one

or both hind limbs, had lower thyroid weights than their non-denervated controls (experiments 1-3; $P = 0.3$ and 0.02 , respectively).

In order to obtain more information about the relation between skeletal musculature and the thyroid gland, a dose-response curve was determined for the effect of thiourea on normal rats, and on rats which had both hind limbs denervated. The experiments were performed very carefully with respect to the grouping by weight.

According to the manufacturer's analysis, dog chow checkers (our common rat food) contains 2.4 parts per million of iodine, which is a relatively high iodine content. Experiments have been carried out (April through May, 1947) in which rats have been fed this 'high iodine' diet. In another series rats were used, which had been raised and were kept during the experimental period (October, 1947) on a 'low iodine' diet (9, 10). Male rats, weighing about 50 gm. were kept for 2 weeks on a diet of 1/3 casein¹ and 2/3 gluten-salt diet. The diets were supplemented by vitamins. Food and distilled water were given *ad libitum*. At the end of 2 weeks, the pure gluten-salt diet was given until the rats had a body weight of 120 to 150 gm.

TABLE 2. COMPOSITION OF DIETS AND CALORIC INTAKE WITH FOOD

DIET	FCP	-CP	F-P	FC-
	%	%	%	%
Crisco.....	8		22.2	10.5
Sucrose.....	64	69.6		84.2
Casein ¹	24	26.1	66.7	
Salt Mixt. ²	4	4.4	11.1	5.3
Change of av. body wt. in %/14 days.....	+6.1	+9.9	-0.4	-15.9

¹ Vitamin test casein, General Biochemicals, Inc.

² Salt mixture No. 2 USP XII, General Biochemicals, Inc.

F, fat; C, carbohydrate; P, protein.

In one group of rats, subdivided in groups of 5 to 10, the hind leg muscles were then denervated and the animals treated with thiourea; another group of rats was treated only with thiourea (given by stomach tube) for 14 days. The range of the single daily dose per 100 gm. of body weight was 0 to 40 mg. in .4 ml of water. At the end of the treatment the rats were killed and the thyroid glands were taken out and weighed.

The results are given in figure 2. If the 'high iodine' diet was given, the average weight of the thyroid gland of the denervated and treated rats was smaller than the thyroid weight of the non-denervated rats. Heavier glands were found in denervated than in non-denervated rats, if the rats were fed with the 'low iodine' diet.

The increment in thyroid weight per mg. of thiourea has been calculated from all the single experimental data at the different dose levels. Standard errors, significance and P -values were determined, assuming that the relation between dose and response is linear. To a first approximation the linear relation holds true for the experiments with the 'high iodine' diet. In the experiments with the 'low iodine' diet

³Vitamin test casein. General Biochemicals, Inc.

only the first half of the two curves (covering the dose range from 0 to 20 mg.) can be considered to express an approximately linear relation between dose and response. If the thyroid weights of the non-denervated rats are compared with the thyroid weights of the denervated rats it is evident that during muscular atrophy a significant increase in thyroid weight (increments 0.280 ± 0.061 , 22 rats, and 0.463 ± 0.040 , 36 rats, respectively; $P = 0.01$ to 0.02) occurred with the 'low iodine' diet. A decrease in thyroid weight during muscular atrophy, which was statistically not significant (increments 0.254 ± 0.035 , 24 rats, and 0.185 ± 0.039 , 24 rats, respectively; $P = 0.1$ to 0.2) was observed with the 'high iodine' diet.

DISCUSSION

In all experiments the atrophy has been expressed in terms of $N-D/N$ 100, N and D being the weight of the normal and the denervated gastrocnemius muscle

TABLE 3. INFLUENCE OF FAT, CARBOHYDRATE AND PROTEIN ON MUSCULAR ATROPHY

DIET	NO. OF RATS	AVERAGE WEIGHT OF GASTROCNEMIUS MUSCLE				MUSCLE ATROPHY (100-d)
		Found data		Reduced Data		
		Norma. (N)	Denerv. (D)	Normal (N)	Denerv. (D)	
		mg/100 gm.				
(1) FCP	9	677.1 ± 14.3	339.0 ± 5.2	100.0 ± 2.1	50.1 ± 0.77	49.9
(2) -CP	16	688.0 ± 11.7	343.4 ± 9.4	100.0 ± 1.7	49.9 ± 1.37	50.1
(3) F-P	16	660.4 ± 11.5	324.2 ± 9.5	100.0 ± 1.7	49.2 ± 1.44	50.8
(4) FC-	19	657.9 ± 10.7	366.7 ± 9.8	100.0 ± 1.6	55.8 ± 1.49	44.2

Significance (S) and P -values (4) against (1): $S = 3.4$; $P < 0.01$
 (4) against (2): $S = 2.9$; $P < 0.01$
 (4) against (3): $S = 3.2$; $P < 0.01$
 (4) against $\frac{(1+2+3)}{3}$: $S = 3.2$; $P < 0.01$

respectively. The normal muscles of rats under the different treatments (tables 1 and 3) did not reveal considerable changes in weight (except *experiment 7*). Therefore, the observed changes in atrophy under the different treatments must be caused by the effect of the drugs on the denervated muscle.

From the known facts in the literature it appears that atrophy of the skeletal musculature is closely related to thyroid function and iodine metabolism. The experimental data presented in this paper give further support of this relationship.

It has been found, both by Diaz-Guerrero and Thomson (11) and us, that under influence of thyroxine muscular atrophy is increased. Thiourea, which inhibits thyroxine synthesis, tends to retard the atrophy. Dinitrophenol (DNP) and arsenic had the same effect as thyroxine but the mode of action of the three substances is unknown. Thyroxine might facilitate the breakdown of larger molecules (Selye, 12), while DNP seems to accelerate more directly the combustion. With respect to As_2O_3 , Kampelmann (13), found that traces of As_2O_3 may activate the thyroid gland in rats. The effect of KI, which in a high dose inhibited the denervation atrophy, might be compared with the clinical observation (5), that exophthalmic goiter and the asso-

ciated muscular atrophy disappear after treatment with iodine and subtotal thyroidectomy.

Muscular atrophy is retarded by giving a low casein diet and increased by giving a high casein diet. The result is due neither to differences in the caloric intake nor to differences in changes in body weight. It is obvious that if the normal (N) muscle loses weight proportionally to the body weight, and the denervated (D) muscle less than that, an 'apparent' reduction in muscular atrophy (smaller value for $N-D/N$

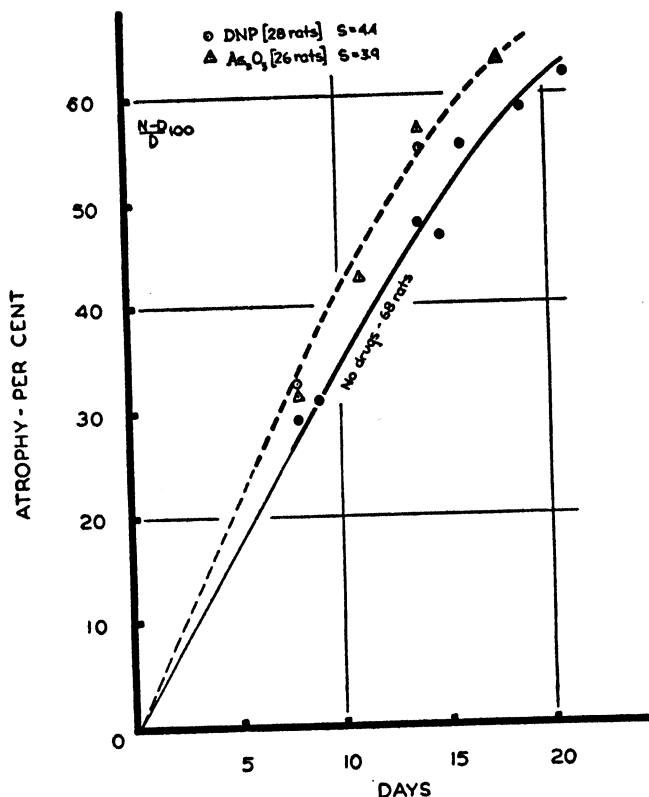


Fig. 2. DEPENDENCE OF WEIGHT of thyroid gland on thiourea treatment of normal and hind-limb denervated rats, which have been fed on either high or low iodine diet.

100) would be observed. We have tried to correlate for each of the 19 rats on protein free diet (mentioned in table 3) the changes in body weight with the degree of atrophy of the gastrocnemius muscle; but such a correlation does not exist. It seems therefore justifiable to conclude that lack of protein in the diet really diminishes muscular atrophy.

This observation parallels early clinical observations (14), in which it was noted that in hyperthyroidism a diet low in protein and high in carbohydrate might depress the increased metabolism to the normal values. Furthermore, Abelin (15) has pointed

out that in the beginning of a state of hyperthyroidism, an increase of the specific dynamic action (due mainly to the administration of protein) brings about an exacerbation of the metabolism in general. None of these observations can be explained satisfactorily since too little is known at present about the biochemical mechanism of the action of the thyroid hormone and its effects on protein metabolism in particular. However, the similar reaction of the denervated skeletal muscle and of general hyperthyroidism to an excess or lack of protein in the diet deserves further attention.

Treatment with thiourea, which increases the weight of the thyroid gland, is more effective in denervated than in normal rats on a 'low iodine' diet. If a 'high iodine' diet is given thiourea seems to be equally or even somewhat less effective on the thyroid glands of rats with denervated hind limbs as compared with normal rats.

The question as to whether or not a difference in iodine content of the food represents the only important factor in these experiments cannot be answered at the moment and only a tentative interpretation can be offered until further studies have been made.

It is known that the skeletal musculature, due to its relative mass, contains the bulk of the iodine in the body. Fellenberg (16), Sturm (17). Morton, Chaikoff and co-workers (18) have shown that extrathyroidal synthesis of thyroxine does take place and that this 'extrathyroidal' thyroxine can be detected in several organs including skeletal muscle. This means that there must be a competition between the thyroid gland and other organs for iodine needed in thyroxine production. The observations reported here could be explained assuming *a*) that thyroxine is needed in muscle metabolism and *b*) that more 'extrathyroidal' thyroxine is needed in denervated than in normal skeletal muscles. In denervated rats with a high iodine intake, the competition for iodine between thyroid gland and other organs is insignificant, and thiourea has little effect on the thyroid gland, since the latter is under the influence of a relatively high extrathyroidal thyroxine production. With low iodine diet, only little extrathyroidal thyroxine might be released from the denervated muscle and, therefore, thiourea would have its full effect on the thyroid.

SUMMARY

Thyroxine, dinitrophenol, and arsenic significantly but slightly increase the rate of denervation atrophy of rat muscles. Thiourea, KI significantly but slightly decrease the rate of atrophy. No effect on the rate of atrophy was observed with iodoacetic acid, mapharsen and BAL. Lack of either fat or carbohydrate in the diet does not effect the rate of atrophy. Lack of protein, however, significantly but slightly decreased the rate of atrophy. The weight of the thyroid gland is influenced by the denervation of the gastrocnemius muscle; thiourea, which increases the thyroid weight, is significantly more effective in denervated than in control rats on a 'low iodine' diet. On a 'high iodine' diet thiourea appears to be equally or less effective in denervated than in control rats.

The experimental results are discussed in relation to the extrathyroidal thyroxine formation in the body. The similarities between the general metabolic alterations in hyperthyroidism and the local changes in denervation atrophy are emphasized.

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ELECTRICAL AND FUNCTIONAL ACTIVITY OF MOTOR NEURONS^{1,2}

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THE importance of electricity in nervous integration is universally accepted, but the source of electrical energy and the manner in which this energy is employed in nervous integration remain debatable problems.

The theory of synaptic transmission of nerve impulses emphasizes the role of transmission of nerve impulses from one neuron to another. It postulates that all parts of the neuron, the dendrites, the cell body and neuraxon conduct nerve impulses by self propagated bio-electrical circuits as illustrated in figure 6.

The theory of neurocellular generation of nerve impulses (1, 2) calls attention to a possible generation of new impulses by the neurons and in contrast questions conduction of nerve impulses by the dendrites and cell body. This theory postulates that a neurocellular current circulates continuously between opposite poles of a neuron in the circuits J, K, L of the schema; that this current generates rhythmic nerve impulses by its outward flow through the neuromembrane in the proximity of the axon hillock; and that variations of intensity of this current importantly control the degree of functional activity of the nerve cell. The current flowing between the poles of the neuron is accordingly designated 'generating current.'

This generating current J, K, L should spread into the neuraxons by electrotonus in the manner of the well known electrotonic spread of artificial polarizing currents (3) as indicated in the lower two schemas of figure 6. This electrotonic spread may fortunately be observed together with the impulse potentials in motor fibers emerging from the spinal cord (4). Thus it becomes possible to subject the theory of neurocellular generation of nerve impulses to a direct test by comparing the number of impulse potentials with the magnitude of the slowly changing electrotonic potentials.

The ventral root of the phrenic nerve of the dog offers several advantages for combined study of electrical and functional activity of spinal motor neurons. It offers a relatively long stretch of motor nerve fibers for application of leading off electrodes; the motor activity of this root is purely inspiratory; the autorhythmicity of the respiratory center provides recurring functional activity of uniform pattern; the details of the functional activity pattern are well known through previous studies on the phrenic nerve and diaphragm (2); the intensity of the functional activity of the

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phrenic neurons can be controlled physiologically without danger of introducing electrical artifacts from electrical stimuli.

Both slowly changing electrotonic potentials and associated quick impulse potentials such as are included in our theory of neurocellular generation of nerve impulses have indeed been recorded by Barron and Matthews (4) in the ventral spinal roots of the frog and cat. These observations will be considered in relation to the present experiments on the dog.

METHOD

Acute experiments were carried out on male dogs (10–20 kg.) anaesthetized with urethane and morphine. The cervical ventral root fibers supplying the phrenic nerve were isolated after exposure by transection of neck muscles and cervical laminectomy. The phrenic nerve was identified at the base of the neck, sectioned, and dissected proximally to the largest contributing ventral cervical root. The ventral root preparation was then placed on two pairs of platinum electrodes mounted in a small lucite moist chamber. These electrodes were separated by 1 cm. The proximal pair served to record the slow electrotonic potentials. The large magnitude of the electrotonic potentials necessitated attenuation by suitable resistors. The distal pair, by means of suitable capacitors, provided free passage of spike potentials without attenuation.

The animal was set up in an electrically shielded box. Electrical activity of the phrenic motor fibers was photographically recorded with the aid of a four stage direct coupled amplifier with push pull pentode pairs and a cathode ray oscilloscope (DuMont 208B). Negative feed back was used in all stages to improve stability and to decrease distortion. A D.C. voltmeter with center zero dial provided an alternate indicating device and means for balancing the amplifier. A loud speaker gave audible indication of action potentials. Respiratory activity was recorded simultaneously with the aid of a rebreathing tank and Hutchinson spirometer.

RESULTS

Electrical Activity of Phrenic Ventral Root During Eupnea. Two distinct types of electrical activity were observed in the ventral phrenic nerve root—a slowly rising and falling negativity with each respiratory cycle and a series of spike potentials superimposed upon the slowly rising wave of negativity (see four sample records in figure 1, and also figures 2, 3 and 4).

The proximal electrode was observed to be negative with respect to the distal electrode, in all phases of the respiratory cycle. As the distance of the proximal electrode from the cord was increased, the slowly undulating negative component was found to decrease. The decrement of the slow component was found to be of the order of 50 per cent reduction per 4 mm. shift of the proximal electrode from the cord. This value is in accord with the findings of Barron and Matthews (4). The spike potentials, however, were unaffected by this procedure. The slowly undulating negative component was therefore interpreted to be electrotonic in nature and the spike component to represent the inspiratory action potentials.

A slowly rising negativity of the proximal electrode would, therefore, indicate an increase of basal negativity of nerve cell bodies with respect to neuraxons. Conversely,

a falling negativity would indicate a decrease of negativity of nerve cells with respect to neuraxons. Rise of negativity of the slow component will be seen to accompany inspiration, and recession of negativity to accompany expiration. Recession of negativity was variable in character, often steep in the early phases of expiration as

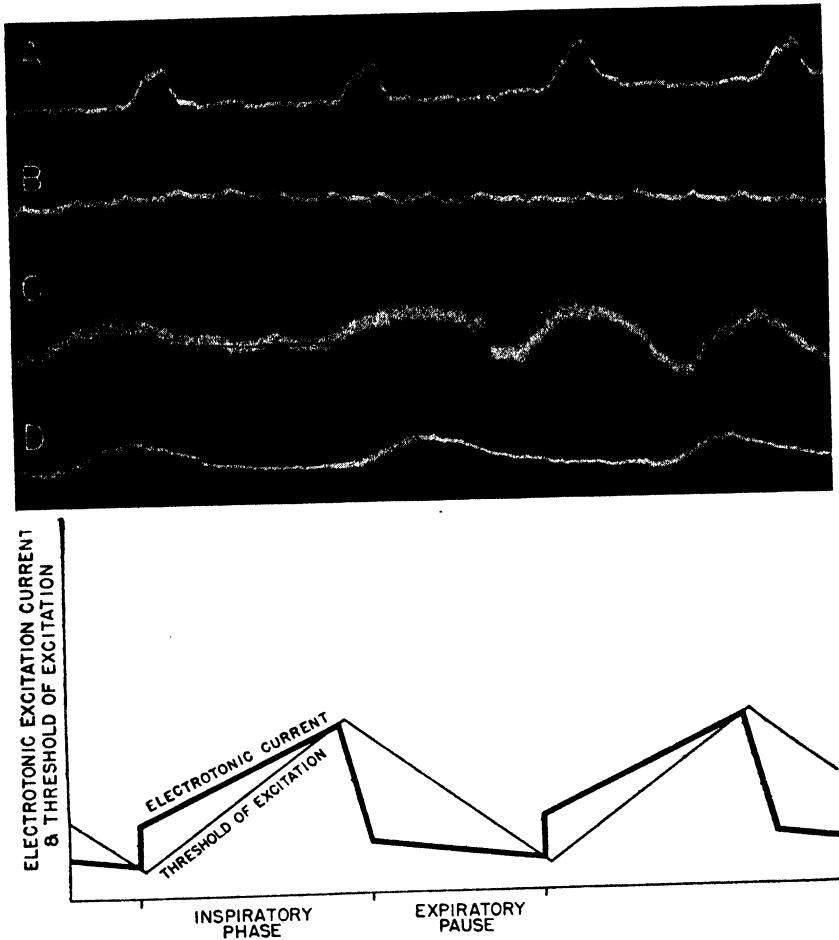


Fig. 1. SAMPLE RECORDS OF ELECTRICAL ACTIVITY of phrenic motor root during eupneic breathing with appended schema of hypothetical mechanisms involved. Note slow undulation of electrotonic negativity and action potentials superimposed on inspiratory fraction. Upstroke of electrotonic potential indicates increasing negativity of phrenic nerve cell bodies with respect to their neuraxons.

compared with the gentle or almost imperceptible recession towards the end of expiration. Gradual recession throughout the expiratory phase, such as seen in figure 1C and 1D, however, was not uncommon.

Rising negativity during inspiration was associated with an increasing number of impulse potentials. This increase of impulse potentials frequently persisted up to the very end of the slow negative rise as in figures 1A; and 2A, B, C, and D. Sometimes impulse potentials terminated before completion of the slow negative rise as

illustrated in figure 1B. Occasionally impulse potentials terminated on the descending limb of negativity as in *record D* of figure 1. Abruptness of termination of impulse potentials was on the whole characteristic whether electrotonic negativity was rising, at its maximum, or falling. Impulse potentials were almost always absent during the phase of expiration despite high electrotonic negativities. It is therefore apparent that the rhythmic discharge of the phrenic neurons cannot be related to intensity of generating currents alone. Other contributing factors playing an important role in nerve cell activity will be considered in the discussion.

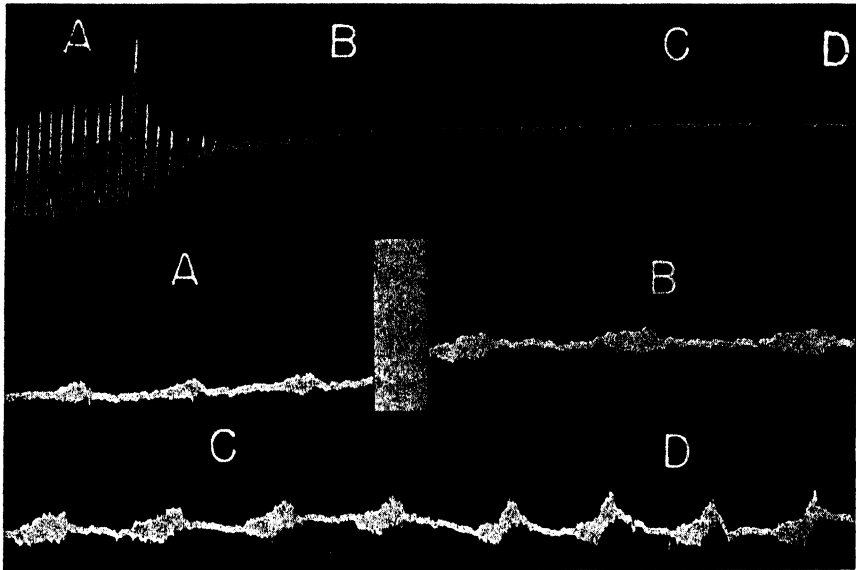


Fig. 2. EFFECTS OF PARALYSIS OF RESPIRATORY MUSCLES on electrical activity of phrenic motor root. The degree of paralysis of the respiratory muscles produced by erythroidine is indicated in the upper tracing of pulmonary ventilation. The change of electrical activity of the phrenic motor root attending paralytic asphyxia is indicated in the lower records A, B, C and D. Note that the gradient as well as the magnitude of the electrotonic potentials increases with prolongation of asphyxia, despite the decrease of strength of contraction of the inspiratory muscles.

Electrical Activity of Phrenic Ventral Root During Paralysis of Respiratory Muscles. It was considered necessary to eliminate electrical artifacts originating in the rhythmical movements of the torso. This was accomplished by intravenous injection of dihydro- β -erythroidine-hydro-bromide (hereafter referred to as erythroidine) in quantity sufficient to produce temporary paralysis of the respiratory muscles. Since there was no artificial ventilation, asphyxial stimulation of the respiratory center is progressive.

The electrical activity of the phrenic motor root during a period of a minute and a half of paralysis is illustrated in figure 2. The upper or respiratory tracing shows the degree of paralysis produced by the injection of erythroidine. *Record A* shows the electrical activity during eupnea immediately before injection and *records B, C and D* show the electrical activity at progressively advanced stages in the course of the

mechanical asphyxia. A progressively increasing functional activity of the phrenic neurons indicated by the increasing number of action potentials agrees with the asphyxial stimulation found by others in curare poisoning (5-8).

Despite a general increase of electrotonic and action potentials during asphyxia, it would appear that the magnitude of the electrotonic potential is temporarily reduced in *record B* at the beginning of asphyxia below that of the control *record A* during eupnea. The results therefore suggest that erythroidine produced two effects on electrotonic spread of current; an immediate reduction, resulting from a direct chemical influence on the neuraxons and a progressive augmentation resulting from an increase of strength of the neurocellular current associated with asphyxial stimulation.

A decreased polarizability of the neuraxons, according to Gotch (3), reduces the spread of a polarizing current. It was therefore suspected that the initial reduction of electrotonic potentials recorded in figure 2B might be due to a decreased polarizability of the phrenic motor fibers. Experiments were done on the vagus nerve of the dog to test this possibility. A uniform polarizing current was sent through the nerve in which circulation was intact. After establishing uniform electrotonic potentials, erythroidine was injected in quantity sufficient to produce respiratory paralysis. The result was a reduction of electrotonic spread of the polarizing current. It is therefore tentatively concluded that the effects of decreased polarizability overbalanced those of increased strength of neurocellular current in the first stages of erythroidine asphyxia in figure 2; whereas, the reverse occurred in the later stages of asphyxia due to the greater increase of intensity of chemical stimulation of respiration. It is also concluded that there is no appreciable spread of current from contracting respiratory muscles to the phrenic ventral root preparation.

Relation of Electrical Activity to Intensity of Functional Activity. If the increase of action potentials accompanying the progressive asphyxia in figure 2 be accepted as index of increasing functional activity of the phrenic neurons, the simultaneous increase of electrotonic potentials indicates a direct relation between functional activity of these neurons and the magnitude of the electrotonic potentials spreading along their neuraxons. Yet it seemed desirable to study this relationship in other ways.

The magnitude of the slow waves of electrotonic negativity of the phrenic root associated with each respiratory cycle was remarkably uniform over long periods of time in animals breathing uniformly. Furthermore, each electrotonic wave was attended by a comparably uniform pattern of inspiratory action potentials (note particularly *record B* of figure 1). In the light of such characteristic uniformity, it seemed especially desirable to determine effects which a variation of intensity of inspiration might have on the electrotonic and impulse potentials.

Normally deepened inspirations occurring sporadically in a series of uniform inspirations were invariably found to be associated with an increase of electrotonic negativity and a corresponding increase of impulse potentials superimposed upon the rising electrotonic negativity.

Inspirations reflexly intensified by cyanide were attended by parallel increase of electrical activity on the phrenic root. Such findings are illustrated in the typical effects of a momentary hyperpnea in figure 3. Comparison of the respiratory tracing

in the upper right corner with the electrical records shows an unmistakable correspondence between strength of inspiration and magnitude of rhythmic electrotonic potentials. Since magnitude of electrotonic potentials is an index of strength of neurocellular currents, it may be tentatively concluded that functional activity of the phrenic neurons is importantly related to the *strength* of these currents. In this connection, it is to be noted that the *rate* with which neurocellular current increased (current-gradient) varied with the depth or strength of inspiration and with the strength of neurocellular current. Current-gradients for inspiration 1, 2, 3, 4, and 5 e.g. were 33° , 56° , 47° , 36° , and 33° , respectively. The percentage increases of current-gradient for respirations 2, 3, and 4 over control respirations 1 and 5 are of

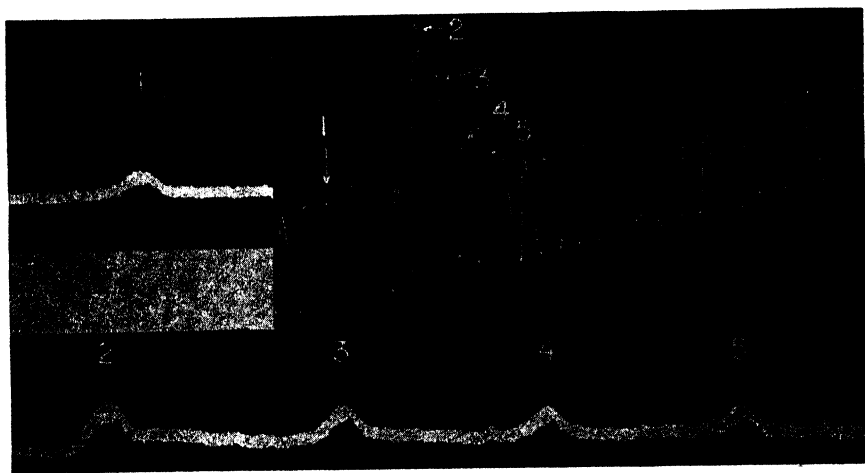


Fig. 3. RELATION OF MAGNITUDE OF ELECTROTONIC POTENTIALS to magnitude of functional activity of phrenic motor neurons during eupnea and hyperpnea. Temporary hyperpnea produced by intravenous injection of cyanide is illustrated in respiratory tracing in upper right corner. Electrical activity of phrenic motor neurons during inspiration 1, 2, 3, 4, and 5, is shown in corresponding electrical records. Note direct relation of magnitude of electrotonic potentials to depth of inspiration. Note also direct relation of potentials gradient to depth of inspiration. For details see text.

the order of 70, 43 and 9 per cent, with the amplification employed. These results are analyzed in the light of the experiments of Lucas (9) and Skogland (10) on current strength and gradient in the discussion.

A similar relation of strength of inspiratory contractions to magnitude of the undulation of electrotonic potentials was found when inspiratory contractions were reduced in strength by artificial overventilation of the lungs and then allowed to recover to normal eupneic strength. In the extremely weak contractions following immediately on overventilation the electrical activities of the phrenic neurons were correspondingly weak (see upper left record of figure 4). The slow electrotonic and the quick action potentials were barely visible. After partial recovery of strength of inspiration in the middle record, both electrotonic and action potentials have recovered substantially. In the lower record where breathing has returned to normal, electrical activities have also returned to normal. As previously noted in figure 3, current-gradient varied with current-strength. The current-gradients were 6° , 14° and

21°, respectively. The current-gradients of the second and third records represent increases of approximately 200 and 300 per cent over the first with the amplifications employed.

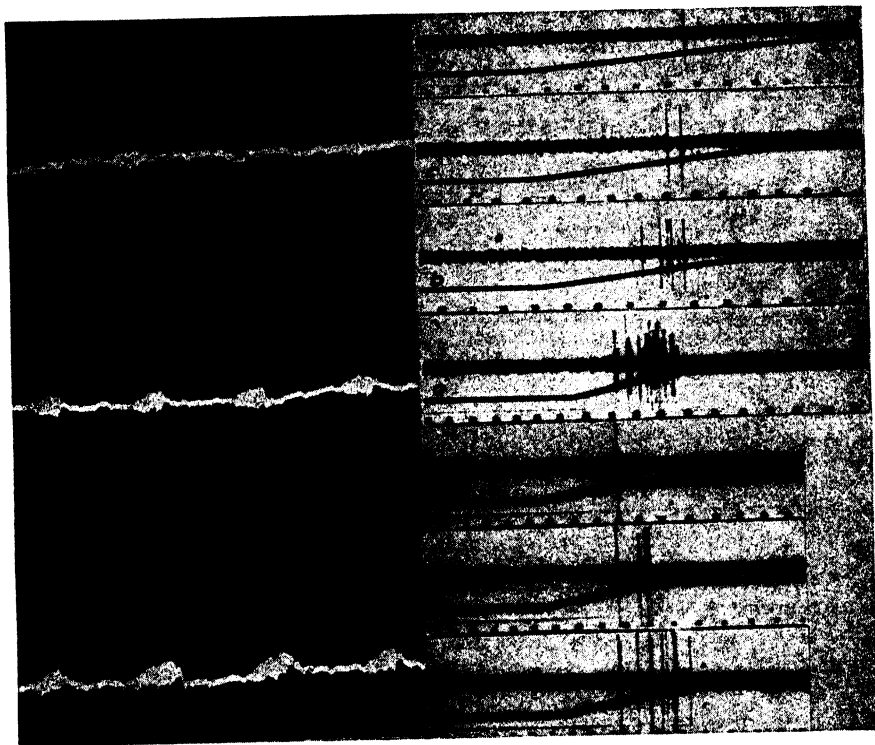


Fig. 4. RELATION OF MAGNITUDE OF ELECTROTONIC POTENTIALS to magnitude of functional activity of phrenic motor neurons during hypopnea and eupnea. The records in the left half of figure show electrical activity of the phrenic motor root during recovery from a preceding artificial hyperventilation of the lungs. Note increase of magnitude and gradient of electrotonic potentials with increasing functional activity of the phrenic motor neurons during recovery from hypocapnia. For details see text.

The records on the right of figure 4 are reproduced from Skogland's paper on "The Response to Linearly Increasing Currents in Mammalian Motor and Sensory Nerves" for convenience of direct comparison with our results on the phrenic motor root. Response to a progressive increase of current-gradient with current-strength maintained constant is seen in the upper series. Response to progressive increase of current-strength with current-gradient maintained constant is seen in the lower series. Note that the response of motor sensory nerves to artificial increase of either current-strength or current-gradient simulates the response of phrenic neurons to combined physiological increase of current-strength and current-gradient.

DISCUSSION

Application of the Theory of Synaptic Transmission of Nerve Impulses to the Functional Activity of the Phrenic Neurons. The means by which impinging impulses are utilized by neurons to control their functional activity is the essence of our problem. Granted that impinging nerve impulses are conducted across the synapse and over the dendrites and cell body to the neuraxon in the manner accepted for

nerve fiber, it follows that reinforcement of inspiratory contraction could result from a numerical increase of individual nerve impulses impinging on and conducted by the phrenic neurons to their respective motor units.

This explanation, however, seems impossible in the light of established principles of neurophysiology. For example, how could this interpretation hold for synaptic transmission of nerve impulses in the recurrent collateral reflex which is one of

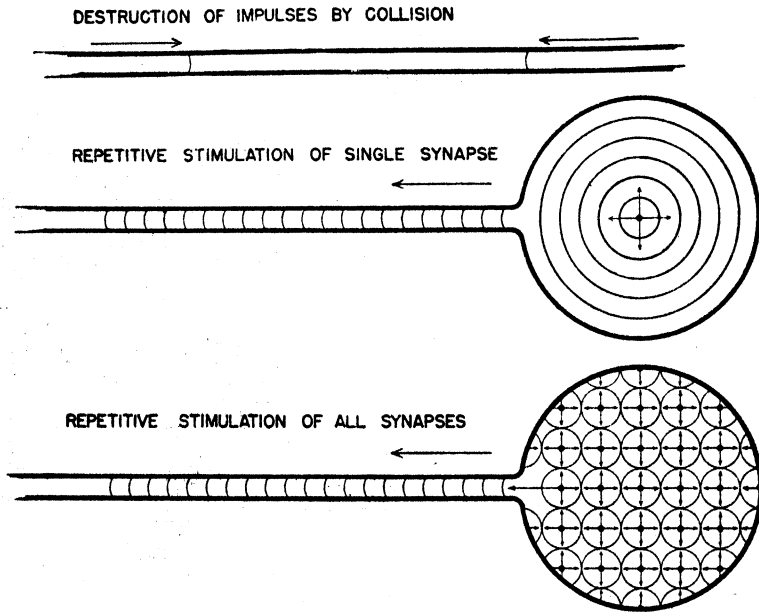


Fig. 5. SCHEMATIZED APPLICATION of principles of neuraxon-function to neuron function.

Schema 1. Mutual destruction of two impulses colliding in a nerve fiber. Destruction is attributable to blocking effect of refractory period in wake of conducted nerve impulses.

Schema 2. Repetitive impingement of nerve impulses on centrally located synapse. Schematized radiate conduction rests on the principle of two directional conduction of the nerve impulse in a nerve fiber. Each electrical wave front reaching the axon hillock is assumed to continue in the neuraxon as a nerve impulse in the manner indicated.

Schema 3. Volley or simultaneous multisynaptic stimulation of neuron. Each impulse initiates radiating nerve impulses. Mutual destruction of most of these impulses is postulated on basis of blocking effect of refractory period in wake of each radiating nerve impulse. Only unopposed dromic wave fronts, namely those reaching the axon-hillock, survive destruction and continue as nerve impulses in neuraxon. This concept leaves the neuron without a mechanism of spatial summation of nerve impulses.

the shortest, if not the shortest, nervous circuits known (approximately 25 microns). Assuming a velocity of impulse conduction of 100, or 10 meters per second, a recurrent impulse would be expected to feed back to the site of its preceding path of conduction in the parent cell in 0.000,0025 and 0.000,025 seconds respectively. Inasmuch as either period is well within the shortest refractory period known, it becomes necessary to find a plausible explanation of how recurrent impulses can function under such conditions.

Whatever the explanation may be, reasonable certainty of non-transmission of these particular impulses must question the possibility of transmission of synaptic

impulses in general. How, e.g., can neurons such as spinal motor, Mauthner and Purkinje cells possessing 500, 10,000, and 1,000,000 synapses respectively, function as transmitters of nerve impulses? By what mechanism could a neuron receiving say 30,000,000 impulses per second transmit these impulses? A schematic application of established principles of axon function to the neuron as a whole will illustrate the need of such enquiry.

Conduction of impulses in either direction by nerve fibers implies a capacity for radial conduction by the neuromembrane of the cell body. Nerve impulses impinging on the relatively broad expanse of the cell body via a centrally located synapse should therefore spread in expanding circles like water waves on a pond. That fraction of each radiating electrical wave which arrives at the axon hillock would continue as a nerve impulse along the neuraxon.

A volley of nerve impulses impinging simultaneously at hypothetically scattered synapses however presents a different picture for the reason that each synaptic potential would be expected to set up its own radiating nerve impulse. Since impulses are destroyed by collision in nerve fibers, it may be inferred that collision of nerve impulses in the neuromembrane of the cell body would produce similar mutual destruction. Those impulses which impinge at the base of the axon hillock would escape blocking on their way to the neuraxon, but they are the only impulses which would be expected to continue on as nerve impulses in the neuraxon. Nevertheless it is a fact that volleys of impulses impinging simultaneously upon a neuron constitute effective stimulation. Such effectiveness depends upon the well known phenomenon of spatial summation.

Similar difficulties encountered in the application of the theory of synaptic transmission will be apparent in figure 6. Assuming simultaneous impingement of two nerve impulses, one at synapse D, and the other at synapse F on the opposite side of the neuron, and picturing transmission as a succession of local bioelectrical circuits such as described by Lillie, it will be seen that impulses D and F arrive at the axon hillock one half-circuit out of step. The earlier arrival of the impulse from synapse F would theoretically destroy the impulse arriving later from the more distant synapse D.

Assume further that impulses D and F are conducted by the dendrite in the conventional manner accepted for nerve fiber. Each impulse would accordingly establish an electrical circuit circumscribing the dendrite in the form of a broad ring travelling in the general direction of the large arrow. On arriving at the cell body each encircling impulse must divide into two impulses; one dromic coursing along the *outer border* of the cell in the general direction of the neuraxon and the other antidromic coursing along the mesial borders of dendrites in the general direction away from the neuraxon.

Application of the Theory of Neurocellular Generation of Nerve Impulses to Functional Activity of Neurons. Inasmuch as the refractory period constitutes an apparently insurmountable obstacle to synaptic transmission of nerve impulses, other means of utilization of electrical energy for functional activation of the neuron must be found. Since synaptic potentials would fuse into neurocellular currents without respect to orderly timing of nerve impulses and of their refractory periods, the theory of neurocellular generation of nerve impulses would seem to avoid difficulties of the theory

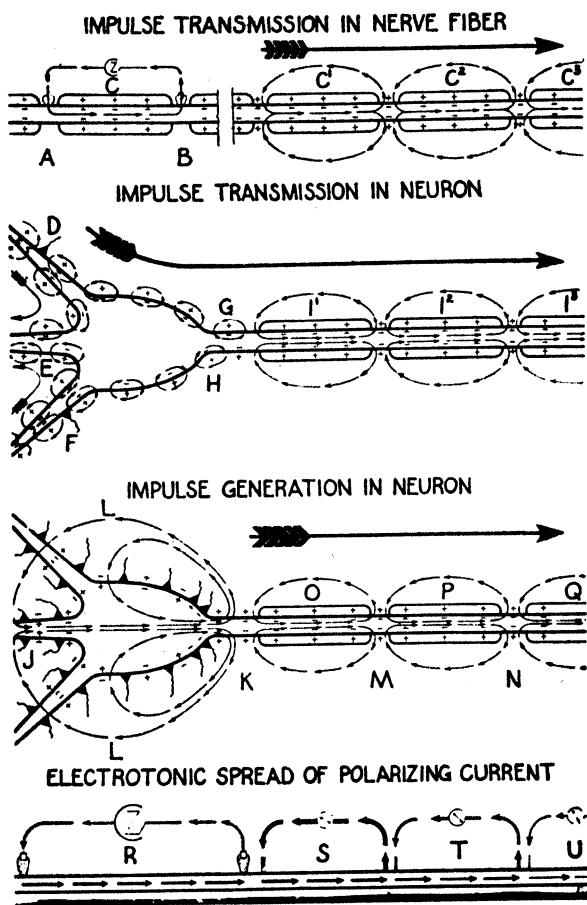


Fig. 6. SCHEMATIC COMPARISON of theory of synaptic transmission of nerve impulses with theory of neurocellular generation of nerve impulses.

Schema 1. Impulse transmission in nerve fiber (8). Polar influence exerted by a constant current passed into a nerve from a battery is shown in left schema. The direction of the current, "positive stream," is indicated by the small arrows. Inhibitory influence at A and excitatory influence at B. The direction of movement of local bio-electrical currents, namely nerve impulse, is represented by large arrow pointing to right in schema. Bio-electrical circuits C^1 , C^2 , and C^3 propagated in myelinated nerve fiber are individually comparable to circuit C at left with anode at one internode and cathode at next internode.

Schema 2. Impulse transmission in neuron. Large arrow illustrates dromic direction of nerve impulses set up at synapses D and F. The bio-electrical circuits are represented by the small arrows. Note that these circuits are represented to be shorter in the regions of the dendrites and cell body than in the neuraxon where myelin segments insure greater length of circuits. Impulses from D and F are out of step at G and in danger

of interference. Only impulses in step with each other would be expected to continue in the neuraxon as represented by the series of bio-electrical circuits r^1 , r^2 , and r^3 . Impulses impinging at D and F theoretically should set up anti-dromic as well as dromic impulses. The antidromic impulses would collide with and destroy dromic impulses coming from the finer dendrites. Furthermore, dromic impulses from synapses D and F coursing along the mesial border of dendrites D and F would be deflected antidromically and join to form a common antidromic impulse on central dendrite E as indicated by the curved arrows between dendrites D and E, and E and F respectively. See text for details.

Schemata 3 and 4. Impulse generation in neuron and electrotonic spread of polarizing current. Neurocellular generating currents are represented as circulating between the poles of the neuron in circuits J, K, L. This current is regarded as a polarizing current which produces electrotonic effects similar to those of an artificial polarizing current flowing through a nerve or nerve fiber. Compare *schemata 3 and 4*. The electrotonic currents set up by the physiological neurocellular current in the phrenic motor root diminish with distance from the neurocellular current just as do the electrotonic currents set up by artificial polarizing currents in a nerve trunk. Electrotonic circuits O, P, and Q may therefore be compared with electrotonic circuits S, T, and U. Generating current J, K, L is held to set up rhythmic nerve impulses in proximity of K. Due to the great strength of electrotonic currents in the immediate proximity of the polarizing current, it is conceivable that impulses could be generated at K, M, N or even more distal internodes. See text for details.

of synaptic transmission of nerve impulses. Synaptic potentials must indeed lose their identity and chronological relation to one another in the process of fusion into

generating currents as molecular impacts do in the generation of steam pressure. And, just as steam pressure is the result of sum total and not of the sequence of molecular impacts, so would strength of neurocellular current be a function of the sum total of synaptic potentials rather than of their sequence.

Theoretical existence of neurocellular currents previously postulated for inspiratory neurons can now be safely accepted as fact. Increase of number of impulse potentials superimposed on a rising electronic negativity may also be regarded as indicative of a direct relation between functional activity of a neuron and the strength of neurocellular currents (figures 2, 3, and 4). Absence of impulse potentials during the expiratory phase despite high electrotonic negativity, however, suggests that strength of neurocellular current cannot be the sole factor determining frequency of neuron discharge. Experiments of Lucas and others would indeed imply that several factors could be involved. The experiments of Lucas on the sciatic nerve of the frog and toad showed effectiveness of current to depend not only on strength but rate of change of current strength. Eight times current-strength sufficient to stimulate at instantaneous increase was found ineffective when current was increased slowly.

Of special significance in the interpretation of our experiments are the studies of Skogland (10) on repetitive response of the sciatic nerve of the cat to artificial galvanic stimulation. Two of Skogland's figures are therefore reproduced in figure 4 for comparison with the response of the phrenic neurons to so-called physiological galvanic currents. The upper of Skogland's series relates to the influence of current-gradient. Current strength was arranged to rise to a constant value in each stimulation. The value chosen corresponded to that which activated a single spike at the slowest gradient. As current-gradient was increased current strength became more effective. The response of the nerve appeared earlier in stimulation, increased to a greater frequency, and lasted for a longer time.

In the lower series of Skogland, current-strength was increased and current-gradient held constant. In *record A* the "current strength of 100 microamp. was just a little above the strength necessary to produce a spike at a latent period of 60 msec. In *B*, at the same gradient, the nerve discharges after the same latent period. But the increase of the stimulus strength from 100 to 124 microamp. has led to three additional responses. The same principle is illustrated in *record C* showing a further increase of current-strength to 160 microamp." (11). It is apparent from the observations of Skogland that increase of current-strength with uniform current-gradient and increase of current-gradient with uniform current-strength, produce analogous increases of effectiveness of stimulation.

The increase of both strength and gradient of the neurocellular currents of the phrenic neurons attending recovery from hypocapnia noted above is therefore of more than passing interest for it calls attention to the possibility of a dual physiological control of neurocellular current in adjustment of functional activity of the phrenic neurons to the requirements of the respiratory act.

Adaptation of nerve fibers to stimulating current as Lucas, Skogland, Solandt, Adrian and Granit (9-13) so clearly demonstrated is a most important factor in repetitive response to continuing excitatory currents. In that connection, it is of interest to refer to the schema appended to figure 1 which was used in the early development of the theory of neurocellular generation of nerve impulses (1, 2). Adap-

tation was represented as a rising curve of 'threshold of excitation' and the generating current was designated as 'excitation current.'

In accordance with this schema it was held: 1) That strength of neurocellular current increased during the phase of inspiration and decreased during the phase of expiration,³ 2) that increase of strength of neurocellular current increased the repetitive response of the phrenic neurons, 3) that the resulting repetitive response of the phrenic neurons increased the threshold of excitation, 4) that rising threshold of excitation overtook the less rapidly rising strength of neurocellular current, 5) that overtaking of neurocellular current by rising threshold terminated repetitive response of the phrenic neurons, 6) that termination of repetitive response produced a steady fall of current of excitation and of threshold of excitation, and 7) that reinitiation of repetitive discharge occurred when decreasing threshold of excitation overtook decreasing strength of excitation current.

According to Skogland, repetitive response of the sciatic nerve to a constant current decreases with time. According to Erlanger and Blair (14) adaptation is greater in motor than in sensory nerves and repetitiousness greater in sensory nerves. Increasing repetitiousness such as occurs in the phrenic neurons with each inspiration would consequently demand the increase of strength and gradient of neurocellular current demonstrated in our experiments.

Normal functional activity of the phrenic neurons would therefore seem to depend upon a nice coordination of strength of neurocellular current and of 'threshold of excitation' to that current, namely 'adaptation.' The experiments of Skogland illustrated in our figure 4 clearly show that an increasing threshold of excitation can overtake an increasing strength of neurocellular current and abruptly terminate inspiratory activity of the phrenic neurons as originally proposed in the schema of figure 1. Note that the response of the sciatic nerve terminates abruptly even though current-strength is still increasing in the upper three records, and that repetitiousness terminates abruptly at maximum sustained intensity of current. A reduction of neurocellular current would therefore not be essential to termination of repetitiousness. The results of Skogland illustrating this point were a welcome clarification to our findings that repetitiousness of the phrenic neurons was occasionally terminated despite the fact that intensity of neurocellular current was increasing (see *records B and C* of figure 1).

The basic similarity of response of the phrenic neurons to physiological changes of neurocellular current on the one hand and of the response of the sciatic nerve to artificially controlled excitatory current on the other hand would seem to support our postulate that the functional activity of the phrenic neurons is importantly controlled by four factors enumerated above—namely current strength, current gradient, membrane excitability and threshold gradient. It is but one more step to apply the principles illustrated in the phrenic neurons to all neurons provided with synaptic drive.

Barron and Matthews have found a correspondence between impulse potentials

³This increase of strength of neurocellular current is attributed to three reflexes: 1) The inspiratory muscle stretch reflex, 2) The inspiratory lung stretch reflex, and 3) The axon reflex of the recurrent collaterals. For details see (2).

and electrotonic potentials in the motor root of the frog and cat such as that described for the phrenic motor root of the dog. They found fewer impulse potentials on the descending limb of electrotonic negativity than on the ascending limb. As a result of their observations, they have come to regard the neuron, not as a conductor of nerve impulses, but as a source of nerve impulses.

Agreement of results coming from different laboratories is indeed satisfying yet it is apparent that our interpretations diverge from those of Barron and Matthews in some respects. We have called attention to polar function of neurons, defined inter-polar electrical circuits, emphasized the role of membrane excitability and in most pertinent contrast have regarded inhibition as a repression of generation of nerve impulses rather than as a repression of conduction of impulses. To the best of our judgment the observations of Barron and Matthews are not contrary to our interpretations. Furthermore we see advantage to a common interpretation of excitation and inhibition based on a common mechanism of *graded generation* of nerve impulses.

SUMMARY

Electrical and functional activity of motor neurons were studied with the aid of a D. C. amplifier. Electrodes placed on the ventral roots of the phrenic nerve in immediate proximity of the cord revealed two forms of electrical activity: slowly undulating waves of negativity in phase with breathing and rapid spike potentials superimposed on the rising limb of negativity. The slowly undulating waves were found to spread by decrement in contrast to the decrementless spread of the spike potentials. The slowly undulating potentials were therefore regarded as electrotonic in nature and were believed to reveal the existence of inter-polar neurocellular currents. The spike potentials were regarded as action potentials produced by the inter-polar currents of the phrenic neuron.

Increase of electrotonic negativity during the inspiratory phase of breathing was associated with a corresponding increase of superimposed action potentials. This was interpreted to indicate a relation of strength of neurocellular currents to generation of nerve impulses. Absence of action potentials during the expiratory phase of breathing despite high electrotonic negativity suggested that strength of neurocellular currents could not be the only important factor controlling repetitive discharge of neurons.

Reasons are presented for believing that the repetitive discharge of the phrenic neurons depends importantly on four key factors: 1) Strength of inter-polar neurocellular currents, 2) rate of change of strength of neurocellular currents, 3) neuro-membrane excitability to these currents, and, 4) changing threshold of neuro-membrane excitability.

It is suggested that the theory of neurocellular generation of nerve impulses circumvents the difficulties of the theory of synaptic transmission of nerve impulses and constitutes a simple mechanism of neuron function compatible with established principles of neuraxon physiology.

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EFFECT OF THE ORAL ADMINISTRATION OF GLUCOSE UPON THE CONCENTRATION OF SERUM AMYLASE IN NORMAL ADULT HUMAN SUBJECTS¹

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IT IS now generally believed that the enzyme α -amylase does not have a rôle in intermediary carbohydrate metabolism although at one time this enzyme was thought to be involved in glycogenolysis. Despite this prevailing view, there are several published reports which indicate that amylase may function in the metabolism of glycogen. In 1924 Cohen (1) made the observation that, following the injection of insulin in dogs, there occurred a decrease in the serum amylase while at the same time the liver amylase increased in concentration. Injection of adrenalin brought about similar changes in the serum and liver amylase values. More recently Taubenhaus and Soskin (2) have shown that in the livers of dogs poisoned with diphtheria toxin glycogenolysis proceeded normally unless the glycogen content was high; in the latter instances, intermediate hydrolytic products of the nature of dextrins were formed, presumably by the enzymatic action of liver amylase. The net effect was an increase in the rate of glycogenolysis despite a decrease in phosphorolysis. There are also conflicting reports as to the effect of clinical diabetes mellitus on the level of serum amylase (3-5). In addition we have observed increases in the serum amylase in alloxan diabetic rabbits which made us think that investigation into the possible relationship of amylase to carbohydrate metabolism might be of interest. To obtain further evidence upon this problem, we have carried out studies of the serum amylase levels during glucose tolerance tests in normal adult human subjects. Urinary amylase excretion studies were also carried out in an attempt to determine whether the serum amylase changes were related to the excretion of amylase in the urine. Serum lipase determinations were made as a reference study of the effect of glucose ingestion upon the level of an enzyme in the serum that is not concerned in carbohydrate metabolism.

EXPERIMENTAL

Oral glucose tolerance tests, using 100 grams of glucose in 250 cc. of water, were carried out on 7 normal adult subjects, 6 male and 1 female. Control experiments in which there was administered 250 cc. of water instead of glucose solution, were carried out on 2 subjects. All tests were made on subjects in the post-absorptive state. In all cases, samples of blood were collected immediately before administration

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of the glucose or water and then one-half hour, 1 hour, 2 and 3 hours later; the urinary bladder was emptied at the time of each blood collection except for an occasional period when the subject was unable to void and the urine was carried over to the next period. The blood samples were analyzed for sugar according to the method of Benedict (6), serum amylase by the technique of Smith and Roe (7), and serum lipase as described by Goldstein, Epstein and Roe (8). Urinary amylase was determined by the same method as serum amylase (7).

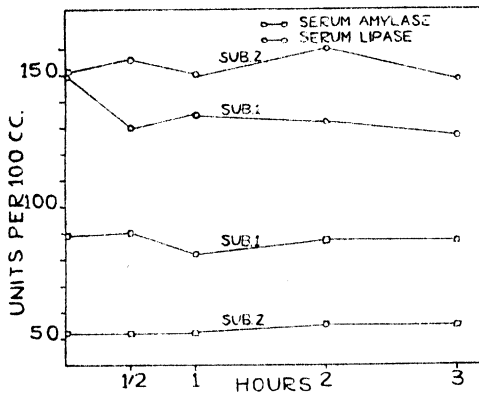


Fig. 1. CURVES SHOWING AMYLASE AND LIPASE VALUES obtained on 2 normal human subjects after the administration of 250 cc. of water to each subject.

TABLE 1. SERUM AMYLASE AND LIPASE VALUES AFTER ORAL ADMINISTRATION OF 100 GRAMS OF GLUCOSE TO NORMAL HUMAN SUBJECTS

SUBJ., SEX, AGE	SERUM AMYLASE					SERUM LIPASE				
	0 hr.	1/2 hr.	1 hr.	2 hr.	3 hr.	0 hr.	1/2 hr.	1 hr.	2 hr.	3 hr.
	Units/100 cc.					Units/100 cc.				
RH, M, 29.....	167	164	143	129	133	79.5	90.5		86	93
NPG, M, 28.....	69	69	54	54	49	68.5	101.5	97	78	78.5
BWS, M, 29.....	78	60	52	40	28	124.5	135	106	98	97
JHE, M, 24.....	79	64	64	60	53	57.5	87.5	82	71	54.5
CRT, M, 36.....	101	89	88	76	65	117.5	112	106	116	94.5
MDR, F, 25.....	78	77	73	62	59	102.5	103	104	108.5	96.5
WK, M, 21.....	59	59	44	36	58	99.5	103	98.5	105	95

RESULTS

The data obtained in the control experiments upon the effect of water administration are shown in figure 1. The amylase curves show practically no change in the serum concentration of amylase after water intake, also a high degree of precision and reproducibility in the experimental procedure. The lipase values are not as free from deviations as the amylase values but the curves indicate that essentially no change in the concentration of serum lipase occurred following the ingestion of water.

The serum amylase and lipase values are shown in table 1. The data show that a marked decrease in serum amylase and no significant change in serum lipase occurred following the administration of glucose.

In table 2 are data showing the urinary amylase excretion before and after the

ingestion of glucose. The average hourly excretion of urinary amylase was determined for control periods of 2 to 6 hours before glucose ingestion. The amylase was determined in each sample of urine collected after administration of glucose and the average hourly excretion after glucose ingestion was calculated. The data show essentially no effect upon urinary amylase excretion resulting from increased glucose utilization.

DISCUSSION

The analysis of variance of the amylase and lipase data is recorded in table 3. Although the major source of variation is among the subjects, with some significant variation due to repetitions, the individual comparison between control and test

TABLE 2. URINARY AMYLASE EXCRETION FOLLOWING ORAL ADMINISTRATION OF 100 GRAMS OF GLUCOSE TO NORMAL HUMAN SUBJECTS

SUBJ.	AVERAGE HOURLY EXCRETION		SUBJ.	AVERAGE HOURLY EXCRETION	
	Before Glucose	After Glucose		Before Glucose	After Glucose
	units	units		units	units
<i>NPG</i>	97.6	95.1	<i>CRT</i>	113.9	113.3
<i>BWS</i>	89.2	75.3	<i>MDR</i>	58.8	66.0
<i>JHE</i>	50.6	50.1	<i>WK</i>	163.0	163.4

TABLE 3. ANALYSIS OF VARIANCE

VARIABLE	SOURCE OF VARIATION	D.F.	MEAN SQUARE	VARIABLE	SOURCE OF VARIATION	D.F.	MEAN SQUARE
Amylase	Subjects	6	5674 ¹	Lipase	Subjects	6	1128 ¹
	Test vs. control	1	1946 ¹		Test vs. control	1	53
	4 Tests	3	568 ¹		4 Tests	3	392 ¹
	Error	24	58		Error	23	65

¹ Highly significant, $P < 0.01$.

values shows that the decrease in amylase concentration is highly significant. For the lipase data the individual comparison between control and test measurements has a mean square value less than error, hence there is no significant change in lipase concentration after glucose administration.

The blood sugar values obtained after glucose administration showed that glucose was absorbed at an apparently normal rate in all subjects. An increased rate of utilization of glucose was therefore present which served as a stimulus to the changes that occurred. The significant change was a marked decrease in serum amylase. This was not due to a loss of amylase in the urine since the data of table 3 show there was no increase in the urinary output of amylase after glucose ingestion. The lack of specific changes in serum amylase and lipase after the administration of water, also the absence of a significant difference between control and test values in the lipase data, favor the point of view that the decrease in serum amylase following glucose ingestion was a specific change due to the presence of extra glucose in the tissues.

The results of our experiments indicate that the amylase of the body tissues may have an important function in carbohydrate metabolism.

SUMMARY

There is a marked decrease in the serum amylase of normal adult human subjects following the oral administration of glucose. The fall in serum amylase is not due to increased urinary excretion of amylase. These results suggest that the amylase of the tissues may participate in carbohydrate metabolism.

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COMPARATIVE EFFECTS OF CARBOHYDRATE, PROTEIN AND FAT WHEN FED AS SINGLE FOODS ON THE SURVIVAL TIME OF RATS UNDER CONDITIONS OF ACCELERATED METABOLISM^{1,2}

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A VAILABLE data indicate that rats may subsist for prolonged periods of time on a diet consisting of a single foodstuff (1-3). Furthermore, physiological interrelationships may frequently be demonstrated far more efficiently under these simplified conditions than when animals are fed a more complete diet. Thus Richter and Rice (4) have demonstrated the specific rôle of thiamine hydrochloride in the utilization of carbohydrate by the rat, and Ershoff (5) has confirmed with the single food choice method the cataractogenic effects of galactose. The method may be employed as a tool useful in indicating possible interrelationships subject to confirmation by other experimental procedures. In the present experiment adult rats were placed on diets consisting of a single foodstuff (either carbohydrate, protein or fat), and their length of survival determined under conditions of accelerated metabolism resulting from (1) thyroxin administration or (2) prolonged exposure to low environmental temperatures.

PROCEDURE AND RESULTS

Thyroxin Series. Sixty-six female rats of the University of Southern California strain were raised to maturity on a stock ration and were selected for the following experiment at approximately 6 months of age and an average weight of 170 grams (range 145 to 210 gm.). The 'single food choice' technique as used by Richter, Holt and others (1-3) was employed. Rats of a standard age and weight were fed diets consisting of a single foodstuff, and the length of survival on the various rations determined. Animals were placed in individual metal cages with raised screen bottoms to prevent access to feces, and sufficient food was administered to assure *ad libitum*

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² These data are from a thesis presented by H. A. Templeton to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.

feeding. Two experimental groups were employed. *Group 1* was given DL-thyroxin³; *group 2* received normal saline solution. The diets employed in each of the groups consisted solely of sucrose, casein⁴ or margarine fat⁵. In addition to the above both groups had fasting controls which had access only to water. For the first 10 days of the experiment DL-thyroxin was administered daily at a level of 100 μ per day; thereafter, it was administered on alternate days in doses of 200 μ until death. *Group 2* was treated in a similar manner except that saline solution was employed in place of the thyroxin. Both thyroxin and saline solution were administered intraperitoneally in a volume of 1 cc. Food consumption was determined on alternate days for each rat during the first 28 days of the experiment.

Results are summarized in table 1. Findings indicate that the survival time of thyroxin-injected rats was significantly less on all diets tested (including fasting controls) than that of animals fed similar diets but administered saline. Furthermore, a marked difference was observed in the relative effectiveness of casein in maintaining survival under the two experimental conditions. In the saline series no significant difference was observed in the survival times of rats fed diets consisting solely of casein, sucrose or margarine fat. In the thyroxin series, however, the survival time of casein-fed rats was significantly less than that of animals fed sucrose or margarine fat.

Data on food consumption indicate that sucrose-fed rats in both the thyroxine and saline series ingested more calories than animals similarly treated but fed casein or margarine fat. A marked reduction was observed, however, in the caloric intake of casein and margarine-fed rats during the first week of thyroxin administration. The caloric intake of animals on these foodstuffs averaged only 42.5 per cent and 39 per cent respectively of that consumed by saline-injected rats on similar rations. No significant reduction in caloric intake was observed following thyroxine administration to sucrose-fed rats. After the first week of feeding food consumption increased in casein and margarine-fed rats in the thyroxine series. By the third week margarine-fed rats in this series were consuming substantially the same number of calories as comparable animals in the saline series. Casein-fed rats in the thyroxin series died before any appreciable increase in caloric consumption had occurred.

Cold Room Series. Sixty-four female rats of the U.S.C. strain were raised to maturity on a stock ration and were selected for the following experiment at approximately 8 months of age and an average weight of 244 grams (range 191 to 270 gm.). Two experimental groups were employed. In *group 1* animals were kept continuously in a large walk-in refrigerator at a temperature of $2 \pm 1.5^{\circ}$ C.; in *group 2* animals were maintained under standard laboratory conditions at a temperature of $23 \pm 2^{\circ}$ C. The diets employed in each of the groups consisted of sucrose, casein, margarine fat or cottonseed oil (Wesson). In addition to the above both groups had fasting controls which had access only to water. Animals were placed in individual metal cages with raised screen bottoms to prevent access to feces and were fed the above

³ Thyroxin (Synthetic Cryst.), Roche-Organon, Inc., Nutley, N. J.

⁴ Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

⁵ Best Foods, Inc., New York, N. Y. The margarine was melted, and the water and protein separated by centrifugation. The fat was poured off and mixed to give a homogenous sample.

diets *ad libitum* until death. Food consumption was determined on alternate days for each rat during the first 28 days of feeding.

Results are summarized in table 2. Findings indicate that the survival time of rats under cold room conditions was significantly less on all diets tested (including fasting controls) than that of animals maintained under room temperature conditions. Furthermore, a marked difference was observed between groups 1 and 2 in the relative effectiveness of the various foodstuffs in maintaining survival. In the room temper-

TABLE 1. COMPARATIVE EFFECTS OF SUCROSE, CASEIN AND MARGARINE FAT WHEN FED AS SINGLE FOODS ON THE SURVIVAL TIME AND CALORIC INTAKE OF RATS ADMINISTERED DL-THYROXIN OR SALINE

DIET	NUMBER OF ANIMALS	INDIVIDUAL SURVIVAL TIME (DAYS)	AVERAGE SURVIVAL TIME (DAYS) ¹	AVERAGE FOOD INTAKE PER RAT (CALORIES PER DAY) DURING FOLLOWING WEEKS OF EXPERIMENT:			
				1st	2nd	3rd	4th
Thyroxin Series							
Fasting	6	7, 7, 8, 9, 9, 11, 11	8.5 ± 0.6				
Casein	10	9, 11, 12, 13, 13, 14, 14, 15, 22, 22	14.5 ± 1.3	5.0 (10)	12.1 (3)	14.2 (2)	—
Margarine fat	10	9, 10, 19, 20, 23, 23, 25, 25, 30, 31	21.5 ± 2.3	7.4 (10)	16.9 (8)	22.2 (6)	23.0 (2)
Sucrose	10	23, 24, 26, 26, 26, 26, 28, 30, 31, 35	27.5 ± 1.1	35.4 (10)	37.4 (10)	32.0 (10)	35.6 (3)
Saline Series							
Fasting	6	11, 12, 14, 14, 15, 16	13.7 ± 0.7				
Casein	8	11, 25, 29, 34, 37, 47, 52, 62	37.1 ± 5.4	12.0 (8)	10.6 (7)	13.5 (7)	18.0 (6)
Margarine fat	8	23, 33, 34, 37, 38, 50, 52, 61	47.2 ± 4.4	10.0 (8)	19.4 (8)	24.4 (8)	24.7 (7)
Sucrose	8	13, 46, 53, 54, 59, 62, 65, 72	53.0 ± 6.0	38.8 (8)	28.8 (7)	30.8 (7)	31.4 (7)

The values in parentheses indicate the number of animals which survived and on which averages are based.

¹ Including standard error of the mean calculated as follows: $\sqrt{\frac{ed^2}{n}} / \sqrt{n}$ where 'd' is the deviation from the mean and 'n' is the number of observations.

ature series the average survival time exceeded 30 days on all diets tested in contrast to 11.2 days for fasting controls. No overlapping in survival time occurred between animals fed the various diets and the fasting controls. Furthermore, in agreement with the findings of Richter *et al.* (1-3) no significant difference was observed in the survival times of rats fed diets consisting solely of sucrose, casein, cottonseed oil or margarine fat, although animals receiving casein or sucrose lived somewhat longer than those fed cottonseed oil or margarine fat. In the cold room series, however, at least 50 per cent of the rats fed casein, cottonseed oil or margarine fat died during the survival period of the fasting controls, and the average survival time on these

foodstuffs exceeded by only 5 days that of animals fed no food at all. Rats fed sucrose, however, lived significantly longer than those fed other diets tested.

Data on food consumption indicate that the longer survival of sucrose-fed rats under cold room conditions was correlated with a greater caloric intake than that ingested by animals fed casein, cottonseed oil or margarine fat. Animals fed sucrose also ingested more calories than those fed other diets tested under room temperature

TABLE 2. COMPARATIVE EFFECTS OF SUCROSE, CASEIN, COTTONSEED OIL AND MARGARINE FAT ON THE SURVIVAL TIME AND CALORIC INTAKE OF RATS MAINTAINED UNDER COLD ROOM AND ROOM TEMPERATURE CONDITIONS

DIET	NUMBER OF ANIMALS	INDIVIDUAL SURVIVAL TIME (DAYS)	AVERAGE SUR- VIVAL TIME (DAYS) ¹	AVERAGE FOOD INTAKE PER RAT (CALORIES PER DAY) DURING FOLLOWING WEEKS OF EXPERIMENT:			
				1st	2nd	3rd	4th
<i>Cold Room Series</i>							
Fasting	6	4, 7, 9, 9, 11, 11	8.5 ± 1.0				
Casein	8	4, 5, 8, 8, 9, 13, 15, 16	9.8 ± 1.5	18.8 (6)	39.0 (2)		
Cottonseed oil	6	4, 9, 10, 12, 13, 25	12.2 ± 2.7	24.1 (5)	39.4 (1)	52.0 (1)	
Margarine fat	6	5, 6, 8, 9, 16, 21	10.8 ± 2.4	17.4 (4)	39.2 (2)		
Sucrose	8	5, 9, 13, 18, 20, 21, 22, 25	16.6 ± 2.7	51.2 (7)	58.0 (5)	63.2 (2)	
<i>Room Temperature Series</i>							
Fasting	6	9, 10, 10, 11, 12, 15	11.2 ± 1.0				
Casein	6	32, 38, 44, 51, 53, 53	45.2 ± 3.4	16.0 (6)	21.2 (6)	22.3 (6)	27.1 (6)
Cottonseed oil	6	16, 27, 30, 38, 40, 43	32.3 ± 3.8	22.2 (6)	23.6 (6)	22.4 (5)	18.6 (4)
Margarine fat	6	21, 27, 36, 36, 37, 52	34.8 ± 4.4	22.5 (6)	17.8 (6)	23.0 (5)	23.2 (4)
Sucrose	6	33, 42, 44, 52, 54, 68	48.8 ± 4.6	42.1 (6)	38.0 (6)	34.2 (6)	32.0 (6)

The values in parentheses indicate the number of animals which survived and on which the averages are based.

¹ Standard error of the mean. See footnote 1, table 1.

conditions as well. During the first week of feeding the average caloric intake for each of the foodstuffs tested was substantially the same in both groups. Subsequent to this period, however, surviving animals in *group 1* ingested considerably more calories than those in *group 2* on a similar ration.

Further tests were conducted to determine the effects of prior adjustment to low environmental temperature on subsequent length of survival under cold room conditions on diets consisting of a single foodstuff. Female rats of the U.S.C. strain were raised to maturity on a stock ration and were selected at 6 to 8 months of age

and an average weight of 218 grams (range 190 to 251 gm.) for the following experiment. Animals were placed in a large walk-in refrigerator maintained at a temperature of $2 \pm 1.5^\circ \text{C}$. and for a period of 12 days were fed a stock ration *ad libitum*. Rats that lost more than 30 grams in weight during this period were discarded and not used for subsequent study. Forty-six of the survivors were selected for the following experiment. The diets employed consisted of sucrose, casein, cottonseed oil or margarine fat. A control group had access only to water. Animals were placed in individual metal cages with raised screen bottoms to prevent access to feces and were fed the above diets *ad libitum* until death.

Data on survival are summarized in table 3. Findings confirm those reported above for animals fed casein, sucrose or cottonseed oil, but differ for those fed margarine fat. Rats fed casein or cottonseed oil lived only slightly longer than those fed no food at all. Survival was significantly increased, however, for animals fed

TABLE 3. SURVIVAL TIME UNDER COLD ROOM CONDITIONS OF RATS FED SINGLE FOODS AFTER PRIOR EXPOSURE TO LOW ENVIRONMENTAL TEMPERATURES

DIET	NUMBER OF ANIMALS	INDIVIDUAL SURVIVAL TIME (DAYS)	AVERAGE SURVIVAL TIME (DAYS) ¹
Fasting	6	3, 4, 4, 4, 7, 8	5.0 ± 0.5
Casein	8 ²	0, 6, 6, 12, 12, 14, 15, 15	10.8 ± 1.4
Cottonseed oil	6 ²	0, 9, 10, 11, 12, 34	13.7 ± 2.1
Margarine fat	6 ²	6, 23, 28, 30, 31, 31	25.6 ± 3.2
Sucrose	10	11, 11, 21, 27, 27, 28, 28, 29, 31, 33	24.6 ± 2.5

¹ See footnote 1, table 1.

² Originally 10 rats were in each of these groups. Animals that died during the first 5 days of feeding, however, were discarded and not included in the tabulation of data.

sucrose or margarine fat. Findings indicate that prior exposure to cold did not affect the subsequent survival time of rats on diets consisting solely of sucrose, casein or cottonseed oil. It was apparently effective, however, in prolonging survival time on a diet of margarine fat.

DISCUSSION

Findings of the present experiment indicate that the ability of rats to subsist on a diet consisting solely of casein was markedly impaired by the administration of thyroxin or prolonged exposure to low environmental temperatures. The average length of survival on a diet consisting solely of sucrose or margarine fat was also decreased under these conditions, but the average survival time on these diets was significantly greater than that on casein. Under room temperature conditions, however, no significant difference was observed in animals not injected with thyroxin in the length of survival on any of the foodstuffs employed. On the basis of the short time period involved in the present experiments, it is improbable that vitamin or mineral deficiencies were a major factor in the results obtained, although it is possible that under the unique conditions of the experiment requirements for one or more

nutrients may have been increased to the point that a deficiency may have been produced.

The failure of casein to prolong survival may be associated with factors other than its nutritional value. Holt and Kajdi (3) have shown that survival on a diet consisting solely of casein is a function of maturity, i.e., body weight. It was found that small rats (65 gm.) survived 8.5 days when fed only casein. The survival time of rats 100 to 150 grams was 27 days, and animals of 150 to 250 grams even longer. Holt and Kajdi (3), furthermore, were able to increase the survival time of casein-fed rats by increasing the protein content of the ration fed prior to the start of the single food experiment. Osborne *et al.* (6) observed that rats at two-thirds of their adult weight failed to eat satisfactorily and lost weight for a short period of time when shifted to a diet containing over 50 per cent of the calories in the form of protein. These workers demonstrated hypertrophy of the kidney frequently amounting to 50 per cent on a diet in which protein accounted for more than 50 per cent of the calories. This hypertrophy was first apparent after eight days of feeding and was complete after 28 to 42 days. At 3 and at 5 days no appreciable increase was noted. Holt and Kajdi (3) have suggested that small animals do not have sufficient reserves to adapt themselves to a high protein diet. The 'adaptation' to a high protein diet involves a marked increase in kidney function necessary for the disposal of products of protein metabolism.

The increased metabolism brought about by the administration of thyroxine or by exposure to low environmental temperatures increases the breakdown of body protein. It is not unlikely that this increase in catabolism together with the shift to a diet containing virtually 100 per cent of its calories in the form of protein may tax the excretion capacities of the kidneys beyond their limit, resulting in death. The available data suggest that rats maintained at low environmental temperatures and those administered thyroxin failed to ingest sufficient calories when administered in the form of casein to meet requirements for survival, due possibly to an inability to metabolize larger quantities of protein.

The variation in survival time of margarine-fed rats in the two cold room experiments suggests that an adaptation to low environmental temperatures is necessary for the efficient utilization of this fat. Animals placed on a diet consisting solely of margarine fat with no previous adaptation to low environmental temperatures survived no longer than casein-fed rats. If animals were exposed to a low environmental temperature for 12 days prior to the start of margarine feeding, the time of survival on this foodstuff was significantly increased. No such increase in survival time was noted, however, for rats fed cottonseed oil. The cause for this discrepancy is not readily apparent. When fed with a complete ration, however, no significant difference in nutritive value between margarine fat and cottonseed oil was observed under conditions of low environmental temperature (7).

SUMMARY

Adult female rats were fed purified diets consisting solely of sucrose, casein or margarine fat, and their length of survival determined after 1) thyroxin administration and 2) prolonged exposure to low environmental temperatures.

Rats fed sucrose or margarine fat lived significantly longer after thyroxin administration than those fed casein when these foodstuffs were administered as the sole constituent of the diet. When saline solution was administered in place of the thyroxin, no significant difference in length of survival was observed on any of the diets employed.

Rats fed sucrose or margarine fat lived significantly longer at an environmental temperature of 2° C. following previous exposure to low environmental temperatures than those fed casein, when these foodstuffs were administered as the sole constituent of the diet. At 23° C. no significant difference in survival time was noted on any of the foodstuffs employed.

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HYPOPROTHROMBINEMIA INDUCED IN THE DOG BY SALICYLIC ACID¹

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THE anticoagulant, 3,3'-methylenebis (4-hydroxycoumarin) (Dicumarol⁴), induces a hypoprothrombinemia in various species of experimental animals and man (1, 2). The *in vitro* chemical degradation of the anticoagulant yields salicylic acid quantitatively (3). This laboratory reported that salicylic acid given to rats on a semi-synthetic ration low in vitamin K induces a temporary hypoprothrombinemia which is prevented by the administration of vitamin K (4). These results were first confirmed in man almost simultaneously by Meyer and Howard (5), Shapiro, Redish and Campbell (6), and Rapoport, Wing and Guest (7).

Relatively small amounts of Dicumarol produce marked diminution in the plasma prothrombin levels of dogs (1). However, preliminary studies indicated that the normal mature dog resisted the hypoprothrombinemic effects of massive oral doses of salicylic acid. In this paper it is shown that salicylic acid augments an already existing state of hypoprothrombinemia, as in dogs given Dicumarol and in new-born pups, and causes a hypoprothrombinemia when a marked tendency toward that state is present, as in bile-fistula dogs recovering from a dose of Dicumarol.

METHODS

The techniques employed as well as the rationale of handling the dogs have been given in previous publications (1, 8). Specific citations are made when necessary. In this study, as in all the previous work (9, 10), whole plasma (100 per cent) and the 50, 25, 12.5, and 6.25 per cent plasma concentrations were routinely explored for prothrombin level (or activity). For reasons previously emphasized (1) the data will be restricted to the plasma concentration of 12.5 per cent (1 part plasma, 7 parts saline solution).

EXPERIMENTAL

Tests in Which Salicylic Acid Failed to Induce a Hypoprothrombinemia. In none of the following trials was the prothrombin time prolonged in blood samples withdrawn at 4, 8, 12, 24, 48 hours or several days, after the salicylate compound was fed or

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⁴ Dicumarol is the trademark for the anticoagulant, 3,3'-methylenebis (4-hydroxycoumarin).

injected: a) mature dogs, maintained on the stock ration⁵ were given orally 1 to 5 gm/kg. of salicylic acid daily for periods ranging from 7 to 30 days. Several animals on this experiment succumbed with a characteristic toxic diathesis (11, p. 167); b) three pups aged 6 weeks were maintained on a diet deficient in vitamin K⁶. At monthly intervals they were given single oral doses of 1 gm/kg. of acetylsalicylic acid (aspirin⁷); c) 6 dogs were administered 50 to 100 mg/kg. of sodium salicylate by means of subcutaneous or intravenous injection.

It was suggested that the inactivity of salicylic acid in the dog and the relatively low activity in the rat is due to the rapidity with which it is excreted (11, p. 36). It has also been shown that the hypoprothrombinemia induced by Dicumarol in dogs and rats was greatly increased when urinary excretion was reduced or prevented (12). Therefore, anuria was produced in 2 mature dogs by the subcutaneous injection of 10 mg/kg. of uranium acetate (12) and immediately thereafter 1 gm/kg. of acetylsalicylic acid was fed. The same doses of uranium acetate and acetylsalicylic acid were repeated after 24 hours. Although one of the dogs died exhibiting the toxic symptoms of salicylism (vomiting, stupor, convulsions, coma), no alteration of the prothrombin time in either dog was observed.

Hypoprothrombinemia was induced by the oral administration of a single dose of 4 ml. or two consecutive daily doses of 2 ml. of chloroform. Large oral doses of acetylsalicylic acid, 100 mg. or 1 gm/kg., were given at various times during the period of detectable hypoprothrombinemia. Even when the acetylsalicylic acid was administered at the stage of maximum hypoprothrombinemia (in some dogs chloroform prolonged the prothrombin time of 12.5 per cent plasma from 25 seconds to more than 100 seconds), no alteration in the usual restoration period to normal prothrombin time was observed.

Hypoprothrombinemia Induced in New-Born Pups by Salicylic Acid. The decreased coagulability of the blood and hypoprothrombinemia which is observed in infant humans has attracted wide attention as the so-called 'hemorrhagic disease of the new-born' (13, p. 270). Inasmuch as the hypocoagulability is prevented by the administration of vitamin K, the disorder has been generally attributed to a deficiency of this vitamin. A survey of the prothrombin time in more than 30 pups born of 7 mothers indicated that a counterpart of the human hemorrhagic disease was also detectable in young pups (14).

Blood samples were taken by withdrawing 0.45 ml. of blood by heart puncture into 0.05 ml. of 0.1 M sodium oxalate, without benefit of anesthesia. The pups were sampled at intervals beginning shortly after parturition until they were 7 days old.

It was found that a moderate hypoprothrombinemia was present at parturition; about 12 hours after birth the prothrombin time was somewhat more prolonged (table 1). Normal prothrombin times characteristic of adult dogs were established

⁵ Skim milk powder 40, yellow corn 15, meat scraps 15, wheat bran 10, wheat middlings 10, alfalfa meal 7, bone meal 2 and salt 1.

⁶ Casein 19, cod liver oil 3, cotton seed oil 2, yeast 4, 1:20 liver powder 2, salts 4 and sucrose 66.

⁷ The salicylate chosen for routine use in these studies. Acetylsalicylic acid (aspirin) induced the most drastic hypoprothrombinemia of approximately 30 different salicylate compounds tested in the rat by a standard procedure (unpublished experiments).

within 2 days after birth. Acetylsalicylic acid was given at various intervals in single oral doses of 10, 50, 100 or 200 mg. Pups given 100 mg. or more of acetylsalicylic acid at any time during the first 5 days of life showed a significant diminution of the level of plasma prothrombin (table 2). Furthermore, many of these animals succumbed exhibiting gross hemorrhage into the abdominal cavity or muscle fascia of the limbs. When 200 mg. of acetylsalicylic acid were given to pups more than 5 days old, no prolongation of the prothrombin time was observed.

Hypoprothrombinemia Induced by Salicylic Acid in Dogs Given 3,3'-Methylenebis (4-Hydroxycoumarin). It has been previously reported that the individual hypopro-

TABLE 1. PROTHROMBIN TIME OF 12.5 PER CENT PLASMA OF NEW-BORN PUPS

TIME AFTER BIRTH	TIME, SEC.	RANGE	NO. OF ANIMALS	TIME AFTER BIRTH	TIME, SEC.	RANGE	NO. OF ANIMALS
4 hrs.	42	32-55	9	3 days	26	22-29	5
12 hrs.	49	30-62	6	5 days	32	25-36	6
1 day	37	33-45	5	6 days	28	27-31	7
2 days	25	22-32	11	mature dog	25	21-29	

TABLE 2. REPRESENTATIVE EFFECT OF SINGLE ORAL DOSES OF ACETYLSALICYLIC ACID ON PROTHROMBIN TIME OF 12.5 PER CENT PLASMA OF NEW-BORN PUPS¹

ACETYLSALICYLIC ACID FED, MG.	AGE OF PUP	PROTHROMBIN TIME IN SECONDS OF 12.5% PLASMA				
		At time of feeding	12 hr. after feeding	24 hr. after feeding	48 hr. after feeding	72 hr. after feeding.
10	4 hr.	35	31	26	28	26
50	4 hr.	35	42	32	34	
100	4 hr.	54	57	67	dead ²	
100	2 days	28	49	96	dead ²	
200	2 days	26	41	35	dead ²	
200	3 days	28	41	44	24	

¹ Each line of figures is a series of values obtained with a single animal. ² At autopsy showed gross hemorrhages.

thrombinemic response evoked by a standard dose of the anticoagulant, 3,3'-methylenebis (4-hydroxycoumarin), in a mature dog is relatively uniform (1). The hypoprothrombinemia arising from a dose of 7.5, 10, or 15 mg/kg. of anticoagulant persists for 3 to 5 days. When single or repeated doses of acetylsalicylic acid were given to dogs at any time after a detectable hypoprothrombinemia had already been induced by the anticoagulant, a further increase in the prothrombin time was obtained. This superimposed hypoprothrombinemia could be detected as a) a prolongation of the prothrombin time greater than that obtained with the anticoagulant alone or b) a delay in the restoration of the normal prothrombin time. Representative responses obtained in 6 of the 35 dogs used in these trials are given in table 3. The effect of the acetylsalicylic acid could be detected 24 hours after feeding; but it was impossible to maintain dogs in a state of hypoprothrombinemia by feeding continuous,

even colossal, doses (5 gm/kg.) for more than 4 to 5 days beyond the period of hypoprothrombinemia induced by the anticoagulant alone.

The superimposed hypoprothrombinemia was roughly proportional to the dose of acetylsalicylic acid given. However, the effect was also dependent on the extent of

TABLE 3. REPRESENTATIVE EFFECTS OF ACETYLSALICYLIC ACID ON THE HYPOPROTHROMBINEMIA INDUCED BY DICUMAROL IN MATURE DOGS

MG/KG. FED	PROTHROMBIN TIME IN SECONDS OF 12.5% PLASMA								PRE-TEST NORMAL VALUES
	Days after feeding Dicumarol								
	1	2	3	4	5	6	7	8	
15 mg. Dicumarol (alone)....	44	54	37	26					25 ± 3
15 mg. Dicumarol + 10 mg. acetylsalicylic acid on 2nd and 4th day.....	36	53	64	57	28				
10 mg. Dicumarol (alone)....	47	65	78	39	28				21 ± 4
10 mg. Dicumarol + 10 mg. acetylsalicylic acid on 3rd and 5th day.....	47	80	76	66	35	35	34	23	
10 mg. Dicumarol (alone)....	42	66	47	36					21 ± 4
10 mg. Dicumarol + 100 mg. acetylsalicylic acid on 2nd, 3rd, 4th, and 5th day.....	43	67	71	74	31				
10 mg. Dicumarol (alone)....	38	69	28	22					28 ± 4
10 mg. Dicumarol + 100 mg. acetylsalicylic acid on 0, 1st, and 2nd day.....	54	87	30	22					
15 mg. Dicumarol (alone)....	44	64	65	35					25 ± 3
15 mg. Dicumarol + 1.0 gm. acetylsalicylic acid on 3rd day.....	45	59	63	113	97	27			
10 mg. Dicumarol (alone)....	41	73	45	34					26 ± 3
10 mg. Dicumarol + 1.0 gm. acetylsalicylic acid on 3rd, 4th, and 5th day.....	46	69	48	115	200	200	dead		

hypoprothrombinemia developed by the anticoagulant. Thus when the anticoagulant was given in minimal detectable quantities, acetylsalicylic acid had no effect on the prothrombin time. The minimum detectable dose of anticoagulant, established separately for each animal, is defined as the amount required to evoke a 3- to 7-second prolongation of the 12.5 per cent prothrombin time. The actual dose of the anticoagulant administered to each dog varied from 2 to 5 mg/kg. When single or consecutive daily doses of 10 or 100 mg/kg. of acetylsalicylic acid were given simultaneously with

or after the anticoagulant, the prothrombin time was not prolonged beyond the control values.

*Hypoprothrombinemia Induced by Salicylic Acid in Bile-Fistula Dogs*⁸. Six dogs were deprived of bile through the use of a bile-fistula. The bile fistula was constructed by ligating and dividing the common bile duct, placing a rubber catheter in the gall bladder and leading the catheter to the outside of the body. The bile was collected in a rubber bag which was emptied every 12 to 24 hours as required. Thus the animal was prevented from ingesting any bile, and a continuous free flow of bile from the liver was permitted. By removing the alfalfa meal from the original stock ration, a diet low in vitamin K was obtained.

During the first week following surgery, 2 of the dogs exhibited prolonged prothrombin times. However, routine determinations of the prothrombin time made during 6 months of continued bile deprivation revealed no difference between bile-

TABLE 4. REPRESENTATIVE HYPOPROTHROMBINEMIA INDUCED BY ACETYSALICYLIC ACID IN A BILE-FISTULA DOG

TREATMENT	TIME IN DAYS	PROTHROMBIN TIME OF 12.5% PLASMA	TREATMENT	TIME IN DAYS	PROTHROMBIN TIME OF 12.5% PLASMA	TREATMENT	TIME IN DAYS	PROTHROMBIN TIME OF 12.5% PLASMA
5 mg/kg. Dicumarol fed on 6 day	0 3 6 9 11	25 ± 4 Control value ¹ 23 85 180 54 28	1.0 gm/kg. acetylsalicylic acid fed on 15th day	15 16 17 18	21 40 38 29	1.0 gm/kg. acetylsalicylic acid fed on 19th day	19 20 21	19 21 22

¹ 45 days after duct ligation.

fistula and normal dogs⁹. It seems likely that the original prolongation was due to surgical liver trauma (16).

Administration of large single doses (1 gm/kg.) of acetylsalicylic acid to these animals did not alter the normal prothrombin times. However, it was found that when acetylsalicylic acid was given after the fistula dog had recovered from a hypoprothrombinemia induced by Dicumarol, a distinct hypoprothrombinemia was produced. The effect took place only within the 7-day period following restoration of normal prothrombin levels. A typical experiment is presented in table 4.

⁸ We are indebted to Dr. Otto V. Hibma, Instructor in Surgery, Wisconsin Medical School, for preparing these animals.

⁹ These findings are in contradiction to those of other laboratories. The Iowa group reported that the time of onset of the hypoprothrombinemia varied but occurred on the average of 3 months after the bile-fistula was introduced (15). However, Dr. J. Garrott Allen, Department of Surgery, Billings Hospital, University of Chicago, has informed us that the results of his unpublished experiments on large numbers of bile-fistula dogs maintained for 2 to 3 years are entirely in accord with our data. We have observed during these experiments that 3,3'-methylenebis (4-hydroxycoumarin) induces a more severe hypoprothrombinemia with equivalent doses in bile-fistula dogs than in normal dogs.

DISCUSSION

The hypoprothrombinemic response of the rat to salicylic acid is controlled by the vitamin K intake (4). While a normal rat maintained on a ration low in vitamin K will readily develop hypoprothrombinemia following administration of salicylic acid, the normal dog, under similar conditions, does not exhibit an increase in prothrombin time. A hypoprothrombinemia induced by salicylic acid was obtained in the dog only under specialized conditions: in new-born pups, in bile-fistula dogs and in dogs given Dicumarol. A decreased availability of vitamin K to perform its metabolic function is common to all these states. The existence of a vitamin K deficiency is now accepted in new-born humans and pups and in animals deprived of bile (13, pp. 255, 279). Although the mechanism by which Dicumarol causes a hypoprothrombinemia is not yet proved, it has been postulated that it is through competitive inhibition with vitamin K in the hepatic synthesis of prothrombin (17, 18). Apparently, then, salicylic acid acts when the vitamin K function is impaired; and the effectiveness of its action varies directly with the degree of impairment as reflected by the prothrombin time. It is most effective after large amounts of Dicumarol, less so in pups where the natural hypoprothrombinemia is comparatively slight, and so slight in bile-fistula dogs that a preliminary hypoprothrombinemia from Dicumarol is necessary. The specificity of vitamin K for this reaction is shown by the complete failure of salicylic acid to cause an increased hypoprothrombinemia after general hepatic injury brought about by the administration of chloroform.

Assuming that this action of salicylic acid is concerned with the synthesis of prothrombin, it must take place either at the same step at which vitamin K and 3,3'-methylenebis (4-hydroxycoumarin) are involved or at some subsequent one. The data from the new-born pups and the bile-fistula dogs give no indication as to which is the case. However the tests with the anticoagulant make it appear unlikely that salicylic acid which cannot compete with vitamin K alone can do so in the presence of a compound obviously possessed of a far greater affinity for its substrate than the vitamin. It is probable that any effect of salicylic acid upon the synthesis of prothrombin by the liver is performed by a mechanism somewhat different from that of Dicumarol.

This type of action is, of course, not the only possibility. Increasing consideration is being given to a direct intravascular reaction of both Dicumarol and salicylic acid which produces inhibition of prothrombin. Anson and Mirsky, in their studies on the reversibility of protein denaturation, found that dilute solutions of salicylate denature hemoglobin and state that, "salicylate probably denatures proteins in general" (19). More recently Seegers reported that salicylic acid denatures pure prothrombin preparations (20).

SUMMARY

When given in huge doses salicylic acid or acetylsalicylic acid had no effect on the prothrombin time of normal dogs receiving a natural ration or of dogs maintained for long periods on a synthetic diet low in vitamin K. The prothrombin time remained normal when large quantities of sodium salicylate were given by subcutaneous or

intravenous injection or when acetylsalicylic acid was fed in repeated doses to dogs in whom anuria had been produced by uranium acetate.

After hypoprothrombinemia had already been induced with Dicumarol, large doses of acetylsalicylic acid increased and prolonged the state of hypoprothrombinemia. Hypoprothrombinemia was induced in new-born pups by acetylsalicylic acid during the first week of life. In chronic bile-fistula dogs, acetylsalicylic acid had no effect; however, when normal prothrombin times were restored after Dicumarol had been administered, feeding acetylsalicylic acid induced a temporary hypoprothrombinemia.

Acetylsalicylic acid was without effect on the prothrombin levels of dogs in which hypoprothrombinemia was produced by the hepatotoxin, chloroform. The hypoprothrombinemic action of salicylic acid is dependent upon the state of the vitamin K metabolism of the animal. It is suggested that it may act in a somewhat different manner than Dicumarol.

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INDIVIDUAL AND AGE VARIATION IN METHEMOGLOBIN FORMATION AND REDUCTION IN RABBIT ERYTHROCYTES

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THE influence of age of the animal host on susceptibility to methemoglobinemia is a problem that has received little, if any, attention. Certain aspects of clinical problems such as the methemoglobinemia from ingestion of nitrite-contaminated well water which has been reported only in very young infants (1) suggest that age may be a factor in the development of methemoglobinemia. Species variation in susceptibility to methemoglobinemia and in rate of methemoglobin (MHb) reduction are well known (2, 3) but not so individual or age variation within a species. From this point of view we have investigated the *in vitro* formation of MHb by nitrite in rabbit corpuscles and the reduction of such MHb by various substrates.

EXPERIMENTAL

Procedure. Heparinized blood was used, obtained by cardiac puncture from healthy, male, New Zealand, albino, short hair rabbits bred in this institution. Weanling animals 10 weeks of age provided a source of young rabbit blood, and animals kept in this Laboratory for more than 6 months served as a source of adult blood. Animals were not usually bled at intervals less than 3 weeks. A standard amount of NaNO_2 was added to red cells which having been packed by 30 minutes' centrifugation at 1700 g. were suspended in homologous plasma or normal saline or, when washed cells were used, in a substrate medium. The saline-phosphate buffer used in the latter consisted of equal parts 0.85 per cent NaCl and $\text{M}/10 \text{ Na}_2\text{HPO}_4$ brought to pH 7.4 with HCl . The reduction of MHb was followed in these cells shaken gently at 38° in stoppered paraffin 25 ml. flasks. MHb was determined at 20 to 30-minute intervals and total hemoglobin (Hb) was measured once on each flask with a portable photometer as described by Andrews and Horecker (4). The average rate of MHb reduction in a given animal's cells was derived by computing the decrease in grams per cent MHb between each two MHb determinations on an hourly basis and taking the mean of the values for all samples between the MHb peak and the first sample showing less than 3.0 gm. per cent MHb. Inasmuch as the rate of MHb reduction in unwashed cells increased gradually after the MHb peak was passed, an attempt was made to calculate the maximal rate by computing the rate of MHb reduction in that part of the curve between the peak and 3.0 gm. per cent MHb having the steepest decline over a period of 60 minutes.

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Erythrocytes were washed by diluting with about 8 volumes of sterile 0.85 per cent NaCl and centrifuging after 10 minutes at ambient temperature. After 4 to 6 washings, the cells were packed by 30 minutes' centrifugation at 1700 g. and used or stored overnight in the cold for use the next morning. The term 'MHb cells' applies to erythrocytes washed, as described, after first having been incubated with approximately .15 ml. 1 per cent NaNO_2 per ml. of cells. Time lapse between cardiac puncture and use of the cells was comparable in the two age groups.

Fresh Blood from Weanling and Adult Rabbits. As shown in table 1, it has been

TABLE 1. COMPARISON OF BLOOD FROM YOUNG AND ADULT RABBITS IN REACTION TO STANDARD DOSE OF NITRITE

	PEAK CMHb REACHED	TIME FROM ADDING NI-TRITE FOR CMHb TO RETURN TO 3.0 GM. %	LSd	AV. RATE OF MHb RED'N. FROM PEAK TO 3.0 GM. %	LSd
	gm. %	hr.	hr.	gm. %/hr.	gm. %/hr.
Cells from adult rabbits in normal saline (1)	12.5	4.65		2.58	
Cells from weanling rabbits in normal saline (2)	12.3	3.31		3.53	
Adult minus young		1.34	.60 ¹	-.95	.43 ¹
Cells from adult rabbits in plasma (2)	10.0	3.30		3.26	
Cells from weanling rabbits in plasma (3)	10.8	2.55		4.47	
Adult minus young		.75	.78 ¹ .59 ²	-1.21	.62 ¹

Flasks contain 2.0 ml. packed cells + 2.0 ml. normal saline or homologous plasma + 0.10 ml. 5% glucose + 0.30 ml. 1% NaNO_2 .

¹ Least significant mean difference, $P = .01$.

² Least significant mean difference, $P = .05$.

(1) Mean derived from 44 runs involving 35 rabbits.

(2) Mean derived from 29 runs involving 24 rabbits.

(3) Mean derived from 12 runs involving 12 rabbits.

found that after addition of a standard nitrite dose, the MHb concentration passes the peak and returns to a low level more quickly when the blood sample is from a young than when it is from an older animal. Analysis of these experiments is complicated by the fact that for an undetermined interval two opposite processes are taking place simultaneously, i.e., the formation of MHb by nitrite and the reduction of the MHb by the red cell substrates. Salient features of the curve were selected and their average values determined for the two age groups. The mean differences between old and young were tested for significance by an analysis of variance. The mean difference was as great as the least highly significant mean difference in regard to both the time required for the concentration of MHb to return to a low level and the average rate of MHb reduction. Whether the differences noted are due to more rapid formation of MHb and less prolonged action of nitrite or to more

rapid reduction of MHb in the young animal's cells is not definite from this procedure, but it was found that late in the period of reduction when nitrite action is in all probability ended (maximum rate of reduction was measured 2 to 3 hours after addition of nitrite in plasma and 3 to 4 hours in saline runs), the reduction of MHb was faster in cells from the young animals.

Pooled blood samples from a previously unused group of young rabbits and from 3 groups of adults after treatment with nitrite showed the usual age difference in MHb reversion.

Reaction to Nitrite with Increasing Age. The difference between erythrocytes from

TABLE 2. REACTION TO NITRITE OF ERYTHROCYTES OF MATURING RABBITS

AGE IN MOS.	RABBIT						
	1.	2.	3.	4.	5.	6.	Av.
A. Time required for CMHb to return to 3.0 gm.% (hr.)							
2½	3	2.42	2.75	2.50	4.17	3.42	3.08
4½	4.58	5.5	3.50	2.83	4.42	4.50	4.25
5½	4.42	4.25	3.50	2.58	4.58	4.83	4.08
6	4.17	4.67	3.83	2.83	6.00	6.00	4.58
7	6.00	5.42	4.17	2.75	5.92	5.67	5.00
10½	6.00	6.00	5.92	3.00	6.00	6.00	5.50
B. Av. rate MHb reduction between peak & 3.0 gm.% (gm.%/hr.)							
2½	4.47	5.37	3.42	4.15	2.20	2.69	3.32
4½	3.10	2.15	2.86	2.63	1.94	2.43	2.69
5½	2.07	2.38	2.10	2.70	1.96	1.83	2.36
6	2.29	2.57	2.74	3.70	1.11	1.63	2.34
7	2.21	2.28	2.26	4.12	1.84	2.00	2.45
10½	1.73	1.96	1.66	3.34	1.73	1.53	1.99

Flasks contain 2.0 ml. packed cells + 2.0 ml. normal saline + 0.15 ml. 5% glucose + 0.30 ml. 1% NaNO₂.

young and adult animals was further evaluated by observing the reaction to nitrite at the time of weaning and at intervals thereafter in blood samples of an additional group of rabbits (table 2). Since the results were identical in either a plasma or saline suspension of the cells except that the rate of MHb reduction was uniformly faster in plasma than in saline, only one set of values is shown. The time required for the MHb concentration to return to 3.0 gm. per cent increases, and the average rate (and maximal rate) of MHb reduction decreases steadily in the first 10 months of life. One of the 6 rabbits studied intensively (*rabbit 4*) underwent only slightly, if at all, the changes noted in the other animals but consistently showed rapid MHb reduction and early return of the MHb level to below 3.0 gm. per cent in these tests and in experiments with washed cells (table 5). Three other rabbits run at weaning and 5 months thereafter all showed the characteristic changes on aging.

Blood from Rabbits Less than Two Months Old. Tests carried out on the pooled blood samples of several individuals at different ages between birth and 6 weeks are

summarized in table 3. During the first 2 weeks of life the values obtained become more and more like those encountered in adult animals, in that the time required for the MHb level to return to 3.0 gm. per cent is longer and the rate of MHb reduction is slower than in rabbits at 10 weeks of age. The only tests in which MHb reduction has taken place as rapidly in cells when suspended in saline as when suspended in homologous plasma of the more than 100 runs in which such a comparison has been possible were these with blood specimens of animals one week of age or less. The time required for MHb to revert to Hb is less for newborn cells in plasma than in saline presumably because the MHb peak is lower for cells in plasma.

Washed Erythrocytes. The possibility was considered that the difference between red cells from weanling and adult rabbits might be due to a greater amount of lactic

TABLE 3. ACTION OF NITRITE ON BLOOD FROM RABBITS LESS THAN 10 WEEKS OF AGE

AGE OF ANIMAL HOST	SOURCE OF DATA	CELLS SUSPENDED IN SALINE		CELLS SUSPENDED IN PLASMA	
		Time for C _{MHb} to return to 3.0 gm. %	Average rate of MHb reduction between peak & 3.0 gm. %	Time for C _{MHb} to return to 3.0 gm. %	Average rate of MHb reduction between peak & 3.0 gm. %
		hr.	gm. %/hr.	hr.	gm. %/hr.
24 hours	Pooled blood of 4 rabbits	3.75	2.33	3.08	2.10
60 hr.	Pooled blood of 6 rabbits	4.25	2.43	3.67	2.07
1 week	Pooled blood of 4 rabbits	5.33	1.87	4.50	1.85
8 weeks	Pooled blood of 4 rabbits	>6	.80	3.17	2.31
4 weeks	Average of 2 tests with pooled blood	3.08	2.97	2.92	3.06
6 weeks	of 4 & 3 rabbits in each	3.25	3.08	2.50	3.83

2.0 ml. packed cells + 2.0 ml. saline or plasma + 0.15 ml. 5% glucose + 0.3 ml. 1% NaNO₂ in each flask.

acid in the former since lactate is said to be the principal substrate for MHb reduction in rabbit blood (5). However, a number of lactate determinations (6) showed no correlation between blood lactate level and either age or individual differences in erythrocyte reaction to nitrite. Increasing the lactate level by 50 or 100 mg. per cent did not markedly alter the red cell reaction to nitrite.

In order better to determine whether the differences observed at different ages were due to intrinsic characteristics of the erythrocytes, tests with washed cells were undertaken. Table 4 gives the average of results of several experiments comparing washed erythrocytes from weanling rabbits and those 8 months of age or older. None of these young animals had been used previously, but 12 of the adults were bled for earlier tests. Whether the substrate added to the washed cells is glucose or sodium lactate, the time required after addition of nitrite for the MHb level to return to 3.0 gm. per cent is less and the maximal (and average) rate of reduction is greater in the weanling than in the adult animals. Analysis of variance showed a probability of less than 0.01 that the mean differences shown in the table between young and old would

occur by chance alone. It was also noted that for the 17 animals in these experiments tested at 10 weeks and again at 8 months of age, the time for MHb to disappear invariably increased and the rate of MHb reduction decreased as the rabbits matured.

Pooled blood samples from 12 individuals in each of 3 groups of adult and a group of young rabbits when washed and run with glucose and lactate showed the same difference between young and old with either substrate as that noted previously with pooled unwashed cells.

Individual Variation. From the results in table 2, the reaction to nitrite shown by the erythrocytes of each individual appeared to reflect a constant characteristic of the cells produced by that animal. To further evaluate the reproducibility of the results, the washed erythrocytes of a number of adult rabbits were tested in the routine manner on two to four occasions. Table 5 showing the relative position assumed

TABLE 4. DISAPPEARANCE OF MHb FROM NITRITE TREATED WASHED ERYTHROCYTES OF WEANLING AND ADULT RABBITS

MEDIUM	GROUP	TIME FOR CMHb TO RETURN TO 3.0 GM. %	LSD ($P = 0.01$)	MAX. RATE MHb RED'N	LSD ($P = 0.01$)
		hr.	hr.	gm. %/hr.	gm. %/hr.
Lactate	Adult	2.96		4.20	
	Weanling	1.88		6.24	
	Adult-weanling	1.08	.3445	-2.04	.7042
Glucose	Adult	4.30		3.09	
	Weanling	2.79		4.48	
	Adult-weanling	1.51	.4036	-1.39	.4627

2.0 ml. packed washed erythrocytes + either 2.0 ml. saline phosphate buffer and 0.3 ml. 5% glucose or 2.0 ml. M/4 sodium lactate + 0.3 ml. 1% NaNO_2 in each flask. Means were derived from one run on each of 62 weanling and 42 adult animals.

by these rabbits in regard to the time required for the MHb level to return to 3.0 gm. per cent demonstrates that the order among the rabbits remains constant over a period of months. Thus in only one run on 2 animals (13 and 14) did the relative position vary as much as 4 places, and it differed 3 places in only one run on 3 rabbits (5A, 12 and 14). There is, however, some variation from one test to another, the reasons for which are not known. In the first of these 4 experiments (also taken as the test on these rabbits for inclusion in table 4) the rabbits with an identification number greater than 6 were only 8 months old and may subsequently have undergone further slowing. At what age the alteration in reaction of red cells to nitrite attributed herein to aging comes to an end has not thus far been accurately defined. The relative position of the individuals throughout this series of runs appears equally constant taking either the average or the maximal MHb reduction rate. Glucose was also used in the first three of these tests, but with this substrate the order assumed by the individuals varies considerably from one test to another. The variability from time to time with glucose as compared with lactate might be due to greater lability *in vitro* of the glucose methemoglobin-reducing mechanism. The relative position of

the individuals when lactate was used agreed poorly with the relative order when glucose was added to aliquots of the same cell preparations. The significance of this is questionable in view of the probable *in vitro* instability of glucose utilization. Excellent agreement was obtained, however, in the relative position of the animals when fumarate and lactate were employed as substrates in the fourth of these tests. A group of 11 other rabbits tested with lactate at weaning and again 6 months later did not show good agreement in the relative position of the animals on these 2 dates. However, they revealed the same kind of agreement in the positions of the animals between the second test and a third and fourth made 2 and 5 months later as was demonstrated

TABLE 5. INDIVIDUAL VARIATION IN ABILITY OF RABBIT ERYTHROCYTES TO RECOVER FROM ADDED NITRITE

Date		3/17		10/6		11/16 ¹	
RABBIT	TIME FOR MHb TO RETURN TO 3.0 GM. %	RABBIT	TIME FOR MHb TO RETURN TO 3.0 GM. %	RABBIT	TIME FOR MHb TO RETURN TO 3.0 GM. %	RABBIT	TIME FOR MHb TO RETURN TO 3.0 GM. %
	hr.		hr.		hr.		hr.
12	1 $\frac{3}{4}$	4	1 $\frac{5}{8}$	4	2	5A	1 $\frac{3}{8}$
4	1 $\frac{1}{4}$	12	1 $\frac{1}{2}$	10	2	4	1 $\frac{5}{8}$
14	1 $\frac{1}{2}$	10	2 $\frac{1}{2}$	12	2 $\frac{1}{8}$	10	1 $\frac{5}{8}$
5A	1 $\frac{1}{2}$	3	2 $\frac{1}{4}$	5A	2 $\frac{1}{4}$	12	2 $\frac{1}{8}$
15	2	14	2 $\frac{1}{2}$	15	2 $\frac{1}{2}$	15	2 $\frac{1}{8}$
3	2 $\frac{1}{2}$	15	2 $\frac{1}{2}$	14	3 $\frac{1}{2}$	11	2 $\frac{5}{8}$
1	2 $\frac{3}{4}$	13	2 $\frac{3}{4}$	11	3 $\frac{1}{2}$	14	2 $\frac{1}{2}$
11	2 $\frac{3}{8}$	11	3 $\frac{1}{2}$	9	3 $\frac{3}{8}$	16	3 $\frac{1}{2}$
7	3 $\frac{1}{4}$	1	3 $\frac{1}{2}$	16	4	9	3 $\frac{1}{8}$
8	3 $\frac{1}{2}$	7	3 $\frac{3}{4}$	13	4 $\frac{1}{2}$	13	3 $\frac{3}{4}$
13	3 $\frac{3}{4}$	5	3 $\frac{3}{4}$	7	4 $\frac{3}{8}$	7	4 $\frac{1}{2}$
5	3 $\frac{1}{2}$	8	5 $\frac{1}{2}$	8	6	8	4 $\frac{1}{2}$
Av.	2.61		3.00		3.38		2.86

Flask contents: 20 ml. packed washed erythrocytes + 2.0 ml. M/4 sodium lactate + 0.3 ml. 1% NaNO₂.

¹ Bath temperature was 40.2° C. on this day through fault in thermoregulator.

from time to time by the group in table 5. The latter were always run 12 at a time, but the former were tested in groups of 3 per day.

Differentiation of MHb Formation and Reduction. It was attempted to determine whether MHb formation in intact washed erythrocytes and hemolysates prepared therefrom is faster in cells obtained from weanling animals. Four pooled blood samples, one containing cells from 12 young rabbits and each of the other 3 containing cells from 12 adult animals were washed and tested at room temperature for MHb formation. Since the number of weanling animals available at any time was limited, this experiment was repeated 4 times using different animals for the young group but essentially the same ones in the adult groups each time. Somewhat inconsistent results were obtained in the separate runs on whole cells, but when all the runs available are averaged for the 2 age groups, as shown in figure 1, it appears that MHb is formed at about the same rate in the 2 groups at first and then proceeds to a higher

level in the young animal cells. A small drop from the peak was noted after 5 to 10 hours in the latter. In hemolysates from young rabbits MHb formation took place at a slightly faster rate in the first few minutes but leveled off within a half hour at a concentration lower than that in hemolysates from adults. The maximum MHb level reached in hemolysates was 50 per cent greater than that in whole cells. This discrepancy is in contrast to results obtained in cat and dog erythrocytes¹ in which though MHb formation is relatively slow in whole cells, the level eventually attained equals that in hemolysates.

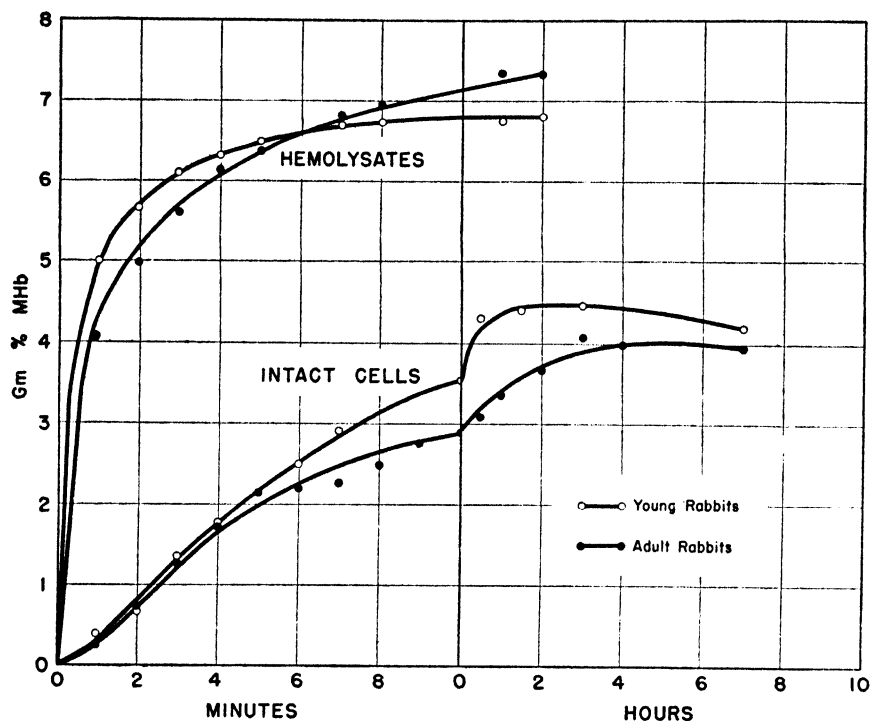


Fig. 1. MHb FORMATION IN HEMOLYSATES and intact cells of weanling and adult rabbits. Flasks contain 1.0 ml. packed washed erythrocytes + 3.0 ml. normal saline or distilled H₂O + .075 ml 1% NaNO₂. Curves represent average of several runs on pooled cells from a number of individuals.

As a measure of the ability of cells to reduce MHb independent of the rate at which it is formed, reduction in 'MHb corpuscles' was studied. The disappearance of MHb in these cells appears to follow the pattern of a first order reaction with both hexoses and metabolic intermediates in rabbit cells since the MHb concentration gave a straight line plotted on a log scale against time. This is in contrast to dog erythrocytes in which reduction occurs as a zero order reaction with the hexoses (7). On the basis of this observation, it was decided that the rate of MHb reduction computed as the average decrease per hour in the log of the MHb concentration, i.e., the first order reaction velocity constant (K_1), could be applied in comparing reduction

¹ Unpublished results

rates in MHb cells from weanling and adult animals. The rate of MHb reduction to the first point below 3.0 gm. per cent in pooled blood of several young rabbits converted to MHb cells was determined for the hexoses and intermediates. This was repeated on another occasion with adult cells. After several such experiments, the conclusion was reached that the rate of MHb reduction is not different in the 2 age groups when glucose, fructose, mannose, or galactose is added but is greater in young than adult when lactate, malate or fumarate is tested. Because a certain degree of daily variation may occur in these tests, an attempt was made to compare with 3 substrates weanling and adult rabbit erythrocytes simultaneously. Here the rate of MHb reduction again was invariably greater in the young than in the adult animals'

TABLE 6. MHb REDUCTION RATES IN MHb CELLS

EXPERIMENT	AGE	GROUP NO.	NO. IN GROUP	POTASSIUM			AV. EXCESS IN YOUNG	
				Fumarate at 1%	Lactate at 1.4%	Lactate at 0.7%	Lactate	Fumarate
				hr. ⁻¹	hr. ⁻¹	hr. ⁻¹	%	%
1	Young	1	12	.357	1.018		18.5	45.7
	Adult	1	12	.216	.950			
	Adult	2	12	.260	.797			
	Adult	3	12	.260	.831			
2	Young	2	12	.444	1.047		33.1	38.3
	Adult	1	12	.335	.784			
	Adult	2	12	.329	.851			
	Adult	4	11	.299	.722			
3	Young	3	12	.399	.863	.682	20.2 & 23.1	60.3
	Adult	1	12	.235	.628	.496		
	Adult	2	12	.242	.833	.601		
	Adult	3	12	.239	.77	.574		
	Adult	4	11	.281	.632	.544		

cells with lactate and fumarate; but no difference was noted with glucose. The positive findings on MHb cells in the second series of tests are summarized in table 6.

If the rate at which MHb is formed has greater bearing than the rate at which it is reduced in determining the time required for the MHb level to return to 3.0 gm. per cent, then the individual variation in rate of formation might be expected to correspond with the variation in time for MHb to disappear. Aliquots of the cells of each animal prepared for the last 2 experiments in table 5 were tested for the rate of MHb formation. It was found that one-third of the animals varied by three or more places in their relative position in the 2 categories. In a preliminary experiment it was attempted to correlate the time for MHb to disappear after nitrite with the rate (K_1) at which MHb is reduced by either lactate or fumarate in MHb cells prepared from these animals. About the same agreement was found here as in the correlation between rate of MHb formation and time to recover from nitrite indicating that the individual differences in time for the MHb to return to 3.0 gm. per cent

after nitrite depend not on either rate of MHb reduction or rate of MHb formation alone, but, more likely, on a combination of both factors.

DISCUSSION

A greater permeability toward substrate could account for both the more rapid disappearance of MHb from erythrocytes of young rabbits after nitrite and the more rapid reduction with lactate, malate or fumarate in MHb corpuscles of the weanling host. The relative inactivity of glucose in MHb corpuscles of young animals, though an apparent contradiction to the permeability thesis, may, in light of the probable untoward effect of nitrite on glucose metabolism (8) and the possible identity of glucose metabolism and permeability in red cells (9, 10) be in the nature of an artefact. Cell permeability to glucose was thought by Engelhardt to determine largely the differences in glycolytic rate in various species (11). In connection with the large individual variation noted here in reaction to nitrite, it should be pointed out that considerable individual variation has been observed for permeability of rabbit erythrocytes to glycerol (12). The cell membrane of reticulocytes has been regarded as less hardened or more sticky than that of mature erythrocytes (13), and the permeability of immature red cells is different from that of the mature cells (12, 14). The number of circulating immature erythrocytes probably influences the recovery from the effect of nitrite in the newborn but would not be expected to do so in rabbits 10 weeks of age (15, 16).

Dziemian, it should be noted, was unable to correlate lipid content of erythrocytes with differences in their permeability due to individual variation of the host or cell immaturity (14). Similarly, we have found no distinct difference in cholesterol, phospholipid or total lipid content of red cells of young and adult rabbits.

Age variation might, on the other hand, be explained on the basis of metabolic activity, the cells of weanling animals containing the limiting enzymes in greater concentrations or having enzymes with a faster rate of turnover. It has been reported, for example, that glycolysis is more rapid in blood of young rabbits (16) and that the red cells of the young of several species contain acid soluble organic phosphates in increased amounts (17). A third possible explanation for the age variation, based on the fact that the rate of alkali-splitting of hemoglobin differs for pigment from infant and adult sources (18), might be that the hemoglobin of weanling animals is particularly susceptible of oxidation and reduction.

Possibly erythrocytes of weanling rabbits contain less of substances other than hemoglobin which react with nitrite. In this event the peak MHb reached in hemolysates (as well as intact cells) should be lower and the time for MHb to disappear should be less in adult rabbits, neither of which was found to be the case.

Were the young cells to contain less of substances which absorb nitrite temporarily and then release it intact as the concentration of nitrite is lowered through its reaction with Hb, then the more rapid disappearance of MHb in young cells might be accounted for. Such a mechanism, though it may occur in the intact cell, cannot be said from the curves of MHb formation to be markedly active in hemolysates. The much slower MHb formation and lower maximal concentration attained in whole cells than in hemolysates appear to indicate that permeability of the cell to nitrite

restricts the reaction with hemoglobin. The fact that MHB formation reaches a somewhat higher level in cells from weanling rabbits could mean that the latter are more permeable to nitrite, particularly considering that a slightly lower MHB level was attained in their hemolysates. In general, the view is favored that erythrocytes of weanling rabbits retain MHB less long than red cells of adult animals after a standard nitrite dose because of greater permeability to both nitrite and substrate.

SUMMARY

Unwashed erythrocytes of adult rabbits suspended in plasma or saline retain MHB for a longer time after addition of a standard nitrite dose *in vitro* than do red cells of weanling rabbits. This age difference applies also to washed erythrocytes supplemented with glucose or lactate. Erythrocytes of rabbits less than 2 months old like those of adults lose MHB relatively slowly. The marked individual variation in time for MHB to disappear from erythrocytes is a constant characteristic after the animals have matured. The individual variation corresponds closely when lactate or fumarate is used as substrate but not when glucose is used. MHB formation after nitrite reaches a slightly higher level in whole cells and a lower level in hemolysates of weanling compared with adult rabbits; and the MHB concentration goes 50 per cent higher in hemolysates than intact cells. MHB reduction in MHB cells supplemented with lactate or fumarate (or probably malate) takes place more rapidly in cells from weanling than in cells from adult animals. Individual variation in time for MHB to disappear after nitrite did not correlate well with individual variation in either rate of MHB formation or rate of reduction in MHB cells. MHB disappears more rapidly from unwashed glucose supplemented red cells when suspended in plasma than when suspended in saline with the exception that this is not true of cells from newborn rabbits.

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WATER AND ELECTROLYTE DISTRIBUTION IN BLOOD AND TISSUES IN NORMAL DOGS FOLLOWING HYPOTONIC SALINE INJECTIONS¹

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THE use of intravenous infusions of hypotonic saline solutions in the post-operative care of patients following major surgical operations has again been recommended lately by Coller *et al.* (1). The major factors considered by these investigators were what they termed the cumulative balances of water, sodium and chloride. Because of the value of such a study, it was considered advisable to investigate further the manner in which the tissues of the body handle large intravenous injections of hypotonic saline solutions.

The following report is based on observations made on 8 normal dogs. Data are presented on the water and electrolyte content of serum, red blood cells and tissues (skeletal muscle, liver, skin and brain), both before and after intravenous injections of large volumes of hypotonic sodium chloride solutions. In addition there are included derived data for the volumes of extracellular and intracellular phases of skeletal muscle and liver, both before and after the injections of the saline. The results from these experiments give further proof that the shift of water across the cellular membranes is the chief means of obtaining osmotic equilibrium in the body fluids, and that the concentration of sodium in the extracellular fluids is one of the most important factors in governing the distribution of water in tissues.

EXPERIMENTAL

Before experimentation, normal dogs, unselected as to breed or sex and weighing 10 to 16 kg., were maintained in metabolism cages for one month or more on alternate daily diets of horse meat and dog chow. The procedure of a typical experiment was as follows: The dog was weighed, placed on an operating board, and anesthetized with intravenous nembutal (25 mg/kg. of body weight). The chest and abdomen were clipped and shaved to remove as much hair as possible. The skin was then washed with distilled water and dried. A cannula was introduced into the femoral artery for continuous manometric (Hg) blood pressure tracings during the experimental period. For the initial analysis, 50 ml. of blood was drawn under oil through a cannula in the other femoral artery. Immediately after withdrawal of the blood, an area of skin about 10 x 8 cm. was dissected and removed from one side of the ventral wall of the abdomen and chest and placed in a glass-stoppered weighing bottle. The rectus abdominis muscle on the same side was then removed and placed in a weighing bottle. All incisions were closed with hemostats.

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The salt solution, warmed to 38°C., was injected by gravity through the femoral vein at a rate of 38 to 40 ml/minute. The injection required from 45 to 50 minutes, after which one hour was allowed to elapse. For the final analysis, a second sample of blood was withdrawn, followed by the removal of skin and rectus abdominis muscle on the opposite side. A sample of liver was then taken and the brain was removed. All tissue samples were placed at once in weighing bottles. The bladder was catheterized for the collection of urine during the entire experimental period.

The study included 8 normal dogs, each of which received an intravenous injection of 170 ml/kg. of body weight of a hypotonic saline solution consisting of 77 mM (0.45 %) NaCl.

CHEMICAL METHODS

The analytical methods have been described and discussed in previous publications (2-5). The serum and defibrinated blood were analyzed for water, chloride, sodium and potassium. Blood cell volume was determined by the use of Van Allen hematocrit tubes without anticoagulant (6). In addition, measurements of the pH, total CO₂ and total nitrogen were made on the serum. The concentrations of the constituents of the red blood cells were calculated indirectly from the values for whole blood and serum and the cell volume. Skeletal muscle and liver were analyzed for fat, blood, water, chloride, sodium and potassium. The amount of circulatory space of the blood in muscle and liver was determined by the spectrophotometric comparison of the amount of hemoglobin in the tissue with that of the whole blood taken simultaneously.

Two grams of minced tissue were transferred to a 25-ml. volumetric glass-stoppered cylinder. Water was added to make to volume and the mixture placed in a 2 to 5° icebox over night. The mixture was then filtered through no. 40 Whatman filter paper into a flask. Two drops of concentrated hydrochloric acid were added while the flask was being shaken. The flask was stoppered and allowed to stand at room temperature for one hour. The optical density of the acid hematin produced was read in a Coleman spectrophotometer at a wave length of 545 millimicra and compared with the density produced from 0.1 ml. blood made to 100 ml. volume with 0.1 N HCl.

The neutral fat of all tissues was extracted as follows: The weighed minced skeletal muscle, liver and small strips of skin that were prepared for water determinations were placed in a 102° oven and dried for 48 hours or to constant weight. The material, in silica beakers, was covered with dry ethyl ether and then placed in a desiccator over ether. The beakers were allowed to stand at room temperature over night, after which the ether was removed with a fine tipped pipette. This process was repeated three times, after which the residues were covered with petroleum ether (b.p. 40-60°) and the beakers allowed to stand in a desiccator over night. After the removal of the petroleum ether, the beakers were placed in a 100° oven for 1 hour, cooled in a desiccator over activated aluminum oxide and weighed. The method of preparation and analysis of skeletal muscle (2, 7), liver (7, 8), skin (9) and brain (10) has been described in detail in previous work.

Calculations for the Derived Data. The volumes of extra- and intracellular phases of muscle and liver were calculated as outlined in the first paper of this series (2). Briefly, the extracellular phase (F) in grams/kg. of tissue,

$$(F) = \frac{(Cl)_T \times (H_2O)_s \times 1000}{1.04 \times (Cl)_s}$$

in which subscripts T and s represent tissue and serum respectively. From the values for (F) the intracellular phase (C) per kilo was estimated by the equation $(C) = 1000 - (F)$. From the values for (C) the intracellular water $(H_2O)_c$ was estimated by the equation $(H_2O)_c = (C) - (S)$ in which (S) represents solids per kilo of blood-free, fat-free tissue.

RESULTS AND DISCUSSION

The analytical results of a typical experiment, both preceding (initial) and following (final) the intravenous injection are presented in table 1. A summary of the averages obtained from the data of all experiments together with the standard deviations are presented in Table 2. These values were used to calculate the derived data for the volume phases of the tissues. The initial normal values for liver and brain (hemisphere and cerebellum) were obtained from previous studies (8, 10). There was little change in arterial pressure during and following the injections in all of the dogs.

Changes in Serum. The changes in the sera of the dogs following the injections consisted of a decrease in bicarbonate, chloride, sodium and total nitrogen, and an increase in water content. The decrease in chloride is about one half as great as the decrease in bicarbonate. The decrease in the sum of bicarbonate and chloride concentrations per kilo of serum water amounted to 14.07 mEq., while the decrease in sodium concentration per kilo of serum water was 17.7 mEq. The lowered protein concentration in the serum accounted for the decreased base-binding capacity of the proteins. It is apparent, therefore, that the decreases in the bicarbonate and chloride content and the base-binding capacity of the proteins, all of which make up the greater part of the total anion content of serum, balance the lowered total base content, mainly sodium.

Changes in Red Blood Cell. In the course of the experiment osmotic equilibrium was adjusted somewhat in the red cells by cellular hydration. The chloride, sodium and potassium concentrations were decreased, although the decrease is not entirely accounted for by hydration. If the values are expressed in units/liter of cell water, the concentration of chloride changes from 90.3 mEq. to 82.5; sodium from 134.8 mEq. to 125.4; and potassium from 11.18 mEq. to 9.92. It is inferred, therefore, that a loss of chloride and sodium occurred from the red cells.

It was of especial interest that in all dogs, the red blood count and hematocrit values changed little following the injection of the large volumes of hypotonic sodium chloride. There was a dilution of the plasma as evidenced by the values for serum water and proteins, as shown in table 2; the original serum water of 92.34 per cent to a final of 94.37 per cent simultaneously with an initial protein of 5.7 gm. per cent to 3.91 gm. per cent. The red cell count and hematocrit values for 5 representative dogs were as follows:

RED CELL AND HEMATOCRIT VALUES

		Initial	Final
Dog 300	Red blood cells, millions/cu. mm.	7.0	6.9
	Hematocrit, per cent	45.5	46.5
Dog 301	Red blood cells, millions	7.3	7.2
	Hematocrit, per cent	50.0	50.0
Dog 308	Red blood cells, millions	7.2	7.5
	Hematocrit, per cent	47.4	49.7
Dog 309	Red blood cells, millions	6.71	6.11
	Hematocrit, per cent	42.5	44.6
Dog 311	Red blood cells, millions	7.17	6.39
	Hematocrit, per cent	50.4	48.4

Examination of the spleen just before the animal was killed disclosed a small, dry, contracted organ, although the animal was under nembutal anesthesia, a condition in which the spleen is usually large and congested (11). Barcroft (12, 13) has emphasized that the contraction of the spleen in a dog serves to discharge blood, from the spleen pulp that is, to cause an autotransfusion and so increase venous return. Later Lewis *et al.* (14) gave experimental evidence that splenic contraction is one of the most important compensatory mechanisms invoked to augment venous return and thus to increase cardiac output. It seems, therefore, from the hematocrit values and the red cell counts that the red cells circulating in the vascular system not only swelled (table 2) but more cells were thrown into the system from the blood reservoirs (15). Also, with an augmented venous return and increased cardiac output, faster osmotic adjustment probably occurred. Results of work on splenectomized dogs will be the subject of a paper now in preparation.

Changes in Tissues. The electrolyte concentration changes in the tissue are regarded as typical of the alterations produced by a relative decrease in extracellular electrolytes accompanied by an increase in total body water. Since osmotic equilibrium between the compartments of a tissue is maintained primarily by the shift of water, the electrolyte concentration changes in the tissues will be considered from the values expressed per 100 grams of tissue solids. The solids are considered as a relative mass of tissue cells. These results before (initial) and following (final) injections of hypotonic sodium chloride are given in table 3. Corrections for free fat and blood were made in muscle and liver, and for free fat alone in skin. Corrections were not made for connective tissue because in short-term experiments on normal dogs the same conclusions could be drawn concerning the distribution of water and electrolytes whether or not connective tissue values were known.

Skeletal Muscle. The increase in muscle water was large, which explains the decrease in concentration of univalent base in muscle water from 162 mEq/liter of water to 141 mEq. The chloride content was increased.

Liver. The injections produced an increase in water and potassium and a decrease in the sodium content of the liver. The final level of the chloride corresponds to the value expected from the lowered serum chloride. The increase in water, therefore, represents a large expansion of the intracellular phase of the tissue.

Skin. The changes in skin involved increases in water, chloride and sodium content. The concentration of univalent base in the tissue water decrease from 167 mEq/liter to 145 mEq. although there was an increase in sodium and no change in potassium. The large increase in water content accounted for the drop in concentra-

TABLE 1. CHANGES IN BLOOD AND TISSUES AFTER INJECTION OF HYPOTONIC NaCl SOLUTION
Solution, 77 mM NaCl. Dog 300, weight 11 kg.; 1870 ml. injected; urine 400 ml.

	pH	CO ₂	H ₂ O	Cl	Na	K	TOTAL N	COLLAGEN N
		mM	gm.	mEq.	mEq.	mEq.	gm.	gm.
<i>Serum—concentration values per kg.</i>								
Initial.....	7.38	23.60	929.2	111.5	142.0	3.88	8.81	
Final.....	7.38	17.88	946.9	108.2	130.0	2.97	5.88	
<i>Blood—concentration values per liter</i>								
	HEMA-TOCRIT	RED BLOOD CELLS						
	%	×10 ⁶ /cu.mm.						
Initial.....	45.5	70	840.0	92.9	121.5	5.71		
Final.....	46.5	6.9	847.7	87.6	114.0	5.58		
<i>Urine—values per liter</i>								
				95.0	85.7			
<i>Muscle—values per kg. of blood-free, fat-free tissue</i>								
Initial.....			767.0	17.35	25.2	94.4		
Final.....			790.0	19.94	28.8	84.3		
<i>Liver—values per kg. of blood-free, fat-free tissue</i>								
Final.....			762.0	42.05	31.64	72.7		
<i>Skin—values per kg. of fat-free tissue</i>								
Initial.....			706.0	98.5	101.0	18.82	46.7	35.0
Final.....			735.0	95.4	92.4	15.31	42.1	31.6
<i>Brain—values per kg. of whole tissue</i>								
Hemisphere, Final.....			785.0	35.10	47.8	90.0	17.5	
Cerebellum, Final.....			770.8	32.24	45.3	90.0	17.2	

tion of univalent base. It is of interest that the total nitrogen and collagen nitrogen values remained the same.

Brain. The changes in the hemispheres and cerebellum involved increased water per 100 grams of solids, explaining the decrease in concentration of univalent base in brain water from 192 mEq. to 178 mEq. in the cerebral hemispheres and 193 mEq. to 179 mEq. in the cerebellum. With the decrease in the concentration of sodium in

TABLE 2. AVERAGE ANALYSES OF SERUM, BLOOD CELLS, AND TISSUES OF ALL DOGS BEFORE AND AFTER INJECTION

Solution: 77 mM NaCl. Values are means and standard deviations.

	pH	CO ₂	H ₂ O	Cl	Na	K	TOTAL N	COLLAGEN N
		mM	gm.	mEq.	mEq.	mEq.	gm.	gm.
<i>Serum—concentration values per kg.</i>								
Initial.....	7.39 ± 0.03	25.50 ± 2.23	923.4 ± 4.5	109.1 ± 1.6	142.5 ± 3.6	4.30 ± 1.07	9.41 ± 0.42	
Final.....	7.40 ± 0.03	18.92 ± 1.76	943.7 ± 3.1	105.1 ± 3.4	128.8 ± 4.1	3.41 ± 0.69	6.56 ± 0.57	
<i>Blood Cells—values per liter</i>								
Initial.....			719 ± 4	64.8 ± 2.1	96.8 ± 3.6	8.03 ± 1.93		
Final.....			727 ± 7	59.9 ± 3.2	91.1 ± 5	7.20 ± 2.78		
<i>Muscle—values per kg. of fat-free, blood-free tissue</i>								
Initial.....			766.4 ± 3.1	20.7 ± 2.2	27.6 ± 4.4	96.4 ± 8.6		
Final.....			789.9 ± 5.8	22.1 ± 3.2	27.3 ± 3.8	83.2 ± 7.6		
<i>Liver—values per kg. of fat-free, blood-free tissue</i>								
Initial ¹			737.3 ± 13.0	35.9 ± 3.8	39.5 ± 4.9	73.2 ± 6.3		
Final.....			767.3 ± 28.0	31.7 ± 5.6	31.2 ± 5.1	72.1 ± 8.8		
<i>Skin—values per kilo of fat-free tissue</i>								
Initial.....			706.7 ± 19.5	89.7 ± 7.4	96.1 ± 7.3	21.36 ± 6.46	47.4 ± 3.8	33.8 ± 4.4
Final.....			755.5 ± 20.8	87.0 ± 5.6	92.2 ± 4.8	17.90 ± 4.48	39.6 ± 3.1	28.3 ± 3.8
<i>Brain—values per kg. of whole tissue</i>								
<i>Hemisphere,</i>								
Initial ²			761.3 ± 8.3	36.7 ± 1.1	51.0 ± 2.4	95.6 ± 4.7	18.9 ± 0.3	
Final.....			778.2 ± 8.5	33.0 ± 1.3	46.4 ± 1.6	91.0 ± 3.5	17.7 ± 0.3	
<i>Cerebellum, Initial²..</i>			745.0 ± 7.0	35.2 ± 0.9	50.8 ± 1.7	92.7 ± 4.0	19.1 ± 0.5	
Final.....			762.6 ± 6.1	30.8 ± 1.5	47.8 ± 2.3	90.6 ± 2.2	17.8 ± 0.4	

¹ Reference 7.² Reference 10.

the serum, in the presence of extra body water, there was no loss of potassium from the brain cells (16). The fact that the chloride concentration was unchanged suggests that the increase in water represents a swelling of the intracellular compartment of the tissue.

TABLE 3. AVERAGE ANALYSES ON DRY-WEIGHT BASIS OF TISSUES OF ALL DOGS, BEFORE AND AFTER INJECTION

	H ₂ O	Cl	Na	K	TOTAL N	COLLAGEN N	Na + K H ₂ O
	gm.	mEq.	mEq.	mEq.	gm.	gm.	mEq/l
<i>Muscle—values per 100 gm. of fat-free, blood-free solid</i>							
Normal.....	328 ± 6	8.87 ± 0.95	11.26 ± 2.02	41.3 ± 3.2			162 ± 12
Hypotonic.....	376 ± 12	10.56 ± 1.76	11.12 ± 2.91	39.6 ± 3.3			141 ± 6
<i>Liver—values per 100 gm. of fat-free, blood-free solid</i>							
Normal.....	280 ± 16	13.72 ± 1.80	15.02 ± 1.86	27.75 ± 2.88			149 ± 13
Hypotonic.....	338 ± 11	13.66 ± 2.07	12.91 ± 1.21	31.20 ± 2.58			134 ± 27
<i>Skin—values per 100 gm. of fat-free solid</i>							
Normal.....	243 ± 28	30.7 ± 2.0	33.0 ± 2.3	7.37 ± 2.09	16.2 ± 0.3	11.5 ± 0.8	167 ± 19
Hypotonic.....	312 ± 37	35.7 ± 2.9	38.0 ± 4.3	7.50 ± 2.22	16.2 ± 0.3	11.5 ± 0.6	145 ± 8
<i>Brain—values per 100 gm. of whole tissue solid</i>							
<i>Hemisphere</i> , Normal....	319 ± 14	15.34 ± 0.79	21.38 ± 1.51	39.9 ± 3.0	7.92 ± 0.18		192 ± 6
Hypotonic.....	351 ± 22	14.91 ± 0.85	21.26 ± 1.11	41.5 ± 1.7	7.97 ± 0.38		178 ± 6
<i>Cerebellum</i> , Normal....	292 ± 14	13.82 ± 0.33	19.90 ± 0.90	36.4 ± 0.8	7.45 ± 0.20		193 ± 4
Hypotonic.....	321 ± 10	12.98 ± 0.62	20.11 ± 1.00	38.12 ± 1.65	7.53 ± 0.25		179 ± 4

Effect of Hypotonic Saline Injections on the Volume Phases of Tissues. Data were derived from the experimental results given in table 2. The calculated mean data for the phase volumes of skeletal muscle and liver from all animals are presented in table 4. The initial phase volumes represent the phases of 1 kilo of muscle from the same dogs before injection. The final volumes represent the phases of 1 kilo of muscle following the injection. To calculate the absolute change in volume in the initial muscle and liver phases, the solid content of the intracellular phase was assumed to be constant. Such a calculation gave the (T)_i values listed.

The absolute mean values obtained for muscle showed that an increase of 111 gm/kg. of control muscle (ΔM) had occurred, of which 44 gm. was attributed to the extracellular phase and 67 gm. to the intracellular phase. The percentage of water in muscle cells increased from 71.8 to 73.8 per cent.

TABLE 4. DERIVED PHASE VOLUME DATA FOR MUSCLE AND LIVER FROM NORMAL DOGS BEFORE AND AFTER INJECTIONS OF HYPOTONIC SODIUM CHLORIDE SOLUTION

T—Extracellular phase plus intracellular phase. (F)—Grams of extracellular phase. $(H_2O)_e$ —Grams of intracellular water. $\{H_2O\}_e$ —Grams of water per kilo of tissue cells. (S)—Solids of intracellular phase. $(T)_t$ —Absolute final weight of the initial phase of tissue after the injection of hypotonic NaCl. Δ —Difference between values before injection (initial) and the absolute values following injection $(T)_t$. All values except $\{H_2O\}_e$ are expressed per kilo of fat-free, blood-free tissue. Solution injected: 77 mm NaCl.

	T	(F)	$(H_2O)_e$	(S)	$\{H_2O\}_e$
	gm.	gm.	gm.	gm.	gm.
<i>Dog 300, muscle</i>					
Initial.....	1000	139	628	233	729
Final.....	1000	167	623	210	748
$(T)_t$	1110	186	692	233	748
Δ	+111	+47	+64		+19
<i>Dog 308, muscle</i>					
Initial.....	1000	190	576	234	711
Final.....	1000	220	574	226	736
$(T)_t$	1136	250	652	234	736
Δ	+136	+60	+76		+25
<i>Mean of muscle from 8 dogs</i>					
Initial.....	1000	169 \pm 18	596 \pm 18	235 \pm 3	718 \pm 7
$(T)_t$	1110 \pm 32	213 \pm 34	663 \pm 28	235 \pm 3	738 \pm 7
Δ	+111	+44	+67		+20
<i>Dog 301, liver</i>					
Initial.....	1000	294	443	263	628
Final.....	1000	294	477	229	676
$(T)_t$	1149	338	548	263	676
Δ	+149	+44	+105		+48
<i>Dog 308, liver</i>					
Initial.....	1000	294	443	263	628
Final.....	1000	258	503	239	679
$(T)_t$	1101	284	554	263	679
Δ	+101	-10	+111		+51
<i>Mean of liver from 8 dogs</i>					
Initial.....	1000	294 \pm 65	443 \pm 67	263 \pm 13	628 \pm 22
$(T)_t$	1152 \pm 65	311 \pm 54	578 \pm 65	263 \pm 13	687 \pm 47
Δ	+152	+17	+135		+59

The absolute mean values obtained for liver showed that an increase of 152 gm/kg. of normal liver (ΔL) had occurred, of which 17 gm. was attributed to the extracellular phase and 135 gm. to the intracellular phase. Thus, in the two tissues, skeletal muscle and liver, in which phase volumes can be approximated, the results obtained after the injection of large volumes of hypotonic saline injections on these phase volumes were as follows: In muscle, about $\frac{2}{3}$ can be attributed to the swelling

of the muscle cells and $\frac{1}{3}$ to an increase in the volume of interstitial fluid; in liver, about $\frac{1}{3}$ can be attributed to the swelling of the liver cells and $\frac{1}{3}$ to an increase in the extracellular phase.

Although we cannot approximate at present the phase volumes in such tissues as skin and brain, some deductions can be made from the concentration of electrolytes observed in these tissues. The large increase in the water of the skin accompanied by slightly lower chloride and sodium concentrations suggests an intracellular edema. If the small increases in water in the hemispheres and cerebellum are significant, the lowered chloride and sodium concentrations indicate that the extra water is intracellular.

Thus, the present data on the phase volumes of tissues under the conditions of intravenous injections of large volumes of hypotonic sodium chloride solutions indicate that a uniform osmotic pressure was produced on the body by a swelling of the tissue cells or, in other words, by an acute intracellular edema.

The average urinary excretion of water, sodium and chloride during the entire experimental period in all dogs amounted to a mean of 437 ± 150 ml. of water, 39.4 ± 14 mEq. of chloride and 39.1 ± 14 mEq. of sodium. These dogs, therefore, excreted a urine with a Na:Cl ratio of 1.0. The urinary loss of water, sodium and chloride was subtracted from the total volume of fluid and the total sodium and total chloride injected respectively in each dog in order to compute the loads of solvent and solute in the infused fluid (17). The load of sodium and chloride was divided by the load of water to obtain the retention concentration. The urine values for 5 dogs are as follows:

DOG NO.	SALINE INJECTION	URINE	WATER LOAD	Na & Cl INJECTED	Cl EXCRETED	Cl LOAD	Na EXCRETED	Na LOAD	RETENTION CONCENTRATION
	ml.	ml.	ml.	mEq.	mEq.	mEq.	mEq.	mEq.	mEq/l.
300	1870	400	1470	143.8	38.0	105.8	34.3	109.5	72-74
302	2750	515	2235	212.0	43.5	168.5	43.0	169.0	75-76
301	2500	315	2285	192.0	34.7	157.3	33.0	159.0	69-69
311	2330	840	1490	179.2	67.0	112.2	65.0	114.2	75-76
315	2300	530	1770	177.0	46.0	131.0	50.6	126.4	74-71

In these animals, following hypotonic saline injections, the retention of a hypotonic salt solution was in agreement with the findings by Coller *et al.* (1) on patients. The retention concentration of sodium chloride in the infused fluid ranged from 60 to 76 mEq/liter. The urine excreted during 120 minutes' time had a concentration of 95.0 ± 12 mEq. NaCl instead of a predicted low concentration of NaCl. Wolf (17) has stated that, generally, when the concentration of infused chloride is less than 102 mEq/liter in concentration, the urine chloride concentration is less than the infused chloride concentration. Although the excretion rate of chloride is ordinarily proportional to the chloride load, the distortion factor and anesthesia must have exerted a greater influence in our animals (table 4).

That sodium concentrations in the extracellular fluids play an important part in the exchange of water between tissue compartments has been demonstrated by many investigators (18-23). The data presented here offer further support to this concept.

In conformity, the decrease in sodium concentration in the body fluids in these experiments was accompanied by a shift of the extra water into the cells in muscle, liver, skin, brain and erythrocytes. By producing a hypotonicity in the extracellular spaces, osmotic equilibrium was maintained by a shift of water into the tissue cells. There is quantitative evidence that the intracellular edema produced in this way occurred in the cells of all tissues studied. It is not known how much swelling the tissue cells can undergo before function is impaired.

SUMMARY

A study was made of the water and electrolyte content and distribution between blood and tissues of the body following the intravenous injection of large volumes of 77 mM sodium chloride solutions (0.45 %).

A lowered extracellular electrolyte concentration in the presence of increased body water was found to be accompanied by a distinct swelling of the intracellular phase of skeletal muscle, liver and skin and the erythrocytes. The data on brain also indicate some swelling of the brain cells or an intracellular edema. The data presented offer further evidence that the concentration of sodium in the extracellular fluids is important in controlling the distribution of water between the fluid compartments of tissues.

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USE OF RADIOACTIVE ISOTOPES TO MEASURE INTRACELLULAR CATION CONCENTRATIONS IN THE NORMAL DOG

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PREVIOUS measurements of intracellular cation concentrations have required desiccation of the animal and the gravimetric determination of total potassium or sodium (1, 2). Correction for the extracellular space and accordingly for extracellular cation has been made on the presumption that chloride is limited to extracellular distribution (3). This same presumption underlies similar determinations in individual organs by the biopsy method (4, 5). The only cells that lend themselves to direct analysis for intracellular cation are the red blood cells (6). It is now clear that chloride is not limited to the extracellular space and that such calculations are invalid (7-10).

In the experiments reported here, extracellular space has been measured with inulin (11, 12). Total body water has been determined with deuterium oxide (13) and total sodium and potassium by the *in vivo* dilution of Na^{24} and K^{42} . These three measurements, when made simultaneously, permit the determination of the average intracellular cation concentration in the living, intact animal.

METHODS

Trained, normal, unanesthetized female dogs in a fasting state were used. A constant inulin infusion was maintained until the inulin was distributed uniformly throughout the extracellular space. The infusion was then stopped and the urine collected until the total inulin in the body had been excreted. The total quantity excreted divided by the inulin plasma concentration at the cessation of the infusion gives the volume of distribution of inulin equal to the extracellular fluid (11, 12). Simultaneously, known amounts of D_2O and Na^{24} or K^{42} were injected intravenously. A blood sample for D_2O analysis was drawn at one and two hours after the injection by which time the D_2O concentration had become constant. The difference between the total body water thus measured and the extracellular volume represents the intracellular volume.

In 8 experiments with radioactive isotopes, arterial blood was taken at frequent intervals over a period of 24 hours to ascertain the time necessary for the plasma specific activity to approach constancy. It was demonstrated that 9 and 3 hours were adequate for K^{42} and Na^{24} , respectively. Urine was collected at periods throughout the experiment to measure the rate of excretion of radioactive isotopes. To calculate

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the total Na or K in the dog, the number of counts retained after the plasma specific activity had become constant was divided by the number of radioactive counts per mEq. of total sodium or potassium in the plasma. The radioactive cation carrier injected did not exceed 1 per cent of the total present in the dog.

Radioactivity was measured with a Geiger-Müller counter with simultaneous readings of decay standard and background. Total sodium and potassium were determined by flame photometry, using an internally compensated Perkin and Elmer Photometer. Inulin was determined by Harrison's method (14) and D₂O by the falling drop method of Keston, *et al.* (15). All samples of K⁴² were purified to separate any contaminating Na²⁴ (16).

RESULTS

As previously reported for these same dogs (11), the extracellular volume averaged 19.4 per cent whereas the total body water averaged 63 per cent of body

TABLE 1. DETERMINATION OF INTRACELLULAR POTASSIUM CONCENTRATION

DOG	INULIN SPACE	HEAVY WATER SPACE	INTRA-CELLULAR SPACE	TOTAL POTASSIUM	EXTRA-CELLULAR POTASSIUM	INTRA-CELLULAR POTASSIUM	INTRA-CELLULAR POTASSIUM CONCENTRATION
	l.	l.	l.	mEq.	mEq.	mEq.	mEq/l.
A	3.800	12.000	8.200	905	13.7	891	109
B	2.800	8.710	5.910	715	11.2	704	119
B	2.700	8.700	6.000	754	14.0	740	123
C	2.740	8.400	5.660	637	11.0	626	111

weight. Table 1 presents simultaneous measurements of extracellular fluid, total body water and total cation. The intracellular cation, as calculated from the difference between total cation and extracellular cation (extracellular volume times plasma concentration) divided by the intracellular water gives the average intracellular cation concentration. In 4 experiments, as shown in table 1, the calculated intracellular potassium concentration averaged 115 mEq/l. of cell water, with values ranging from 109 to 124. Similar sodium measurements in 10 dogs have averaged 35 mEq/l., as shown in table 2. The sum of the intracellular concentrations of sodium and potassium is approximately 150 mEq/l.

In figure 1, the specific activity of the plasma (counts/l. divided by the concentration of total cation in mEq/l.), expressed as counts/mEq., is plotted against time in hours following the intravenous injection of the radioactive isotope. The specific activity after the injection of K⁴² falls rapidly and approaches a constant value at 9 hours. This state of constancy has been considered to be one of practical equilibrium. The specific activity of Na²⁴ shows a much slower and more prolonged decline. Equilibrium after Na²⁴ injection consequently requires at least 24 hours. To obviate the disadvantages of such a lengthy equilibrating period, the 3-hour plasma specific activity has been used for the calculation of total body sodium, although such a measurement represents only about 90 per cent of the total body sodium.

During a 24-hour period urinary loss of K^{42} varied from 2 to 12 per cent of the total isotope injected. This loss was subtracted from the injected dosage in all calculations. Sodium loss was much smaller, amounting to 1 to 2 per cent of the injected counts, which quantity is insignificant in the determination of the 3-hour sodium space. In 2 experiments, the specific activities of Na^{24} in urine and plasma were identical throughout the 24-hour period when allowance was made for urinary delay time (2 to 8 min.) in the formation of urine.

In figure 2 the differences between specific activity at various times and the equilibrium specific activity are plotted on a logarithmic scale against time in hours on an arithmetic scale. In each of the experiments performed with potassium, the data so plotted fall along a straight line. This relation does not hold for sodium.

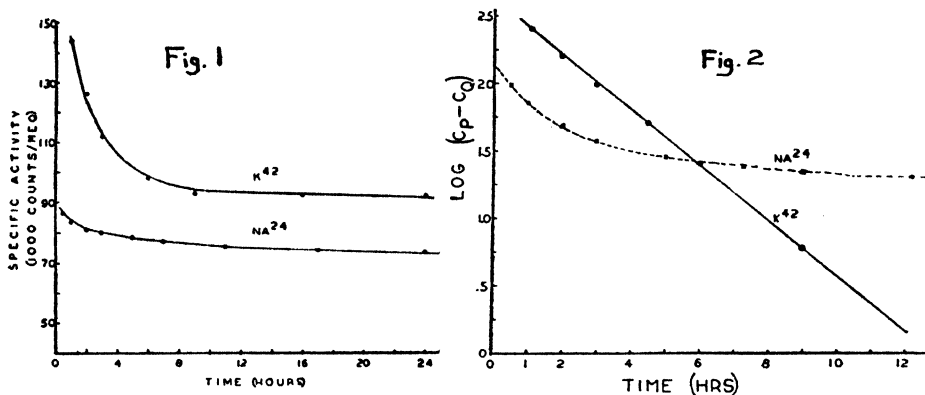


Fig. 1. SPECIFIC ACTIVITY in relation to time following injection of the radioactive isotope.

Fig. 2. LOGARITHM OF DIFFERENCE between specific activity at given times and equilibrium specific activity plotted against time following injection of the isotope.

In table 3, the extracellular fluid volume is compared with volumes of distribution of Na^{24} at 0.5 hour, 3 hours, and 24 hours after injection. Using the 3-hour space as a standard of reference, it is apparent that approximately one-third of the total sodium in the body lies outside the extracellular space. Actually even the half-hour sodium space is significantly larger than the inulin space. Conversely, no more than 2 per cent of the total potassium is located in the extracellular compartment (table 1).

DISCUSSION

Figure 1 demonstrates the great rapidity with which potassium leaves the extracellular compartment; 70 to 80 per cent of the final equilibrium specific activity is attained within 3 hours. Actually, visceral organs like the liver and kidney acquire K^{42} so rapidly that temporarily the specific activity of these organs is higher than that of plasma (17). This is not true for the vast bulk of body cells in muscle, brain and skin, in which equilibrium distribution is more slowly attained (18). Previous conclusions that K^{42} does not enter cells completely (19) is probably the result of contamination of the K^{42} with small amounts of Na^{24} . It has been estimated that a

sodium contamination so small as 0.01 per cent may cause a 100 per cent error in the calculation of radioactivity attributable to K^{42} (18).

The circumstance that the data from a typical K^{42} experiment, as plotted in figure 2, yields a straight line indicates that only one rate of exchange is involved in the movement of potassium between the plasma and the intracellular compartment through the extracellular space. Extracellular and intracellular potassium are therefore in constant interchange with each other at approximately the same rate in different body tissues.

Sodium²⁴ data plotted as above fall along a constantly changing curve (fig. 2). This is in keeping with the observations of Gellhorn *et al.* (20) who demonstrated that the disappearance of Na^{24} from the plasma of the dog followed a double exponential curve. These investigators postulated that this double component was dependent upon 2 different rates of transfer of sodium across the capillary membrane in different parts of the body. However, it seems unlikely that potassium passes through the vas-

TABLE 2. DETERMINATION OF INTRACELLULAR SODIUM CONCENTRATION

DOG	INULIN SPACE	HEAVY WATER SPACE	INTRA-CELLULAR SPACE	TOTAL SODIUM	EXTRA-CELLULAR SODIUM	INTRA-CELLULAR SODIUM	INTRA-CELLULAR SODIUM CONCENTRATION
	<i>l.</i>	<i>l.</i>	<i>l.</i>	<i>mEq.</i>	<i>mEq.</i>	<i>mEq.</i>	<i>mEq./l.</i>
<i>D</i>	1.930	6.100	4.170	480	295	135	41.0
<i>A</i>	3.670	12.200	8.530	802	568	234	27.5
<i>E</i>	2.910	9.050	6.140	692	445	247	40.2
<i>F</i>	3.880	13.000	9.120	912	590	322	35.3

cular membrane at one rate but that sodium does so at two different rates. Further, since the capillary wall is so permeable to sodium, it is improbable that this membrane will be the limiting factor in determining the rate of disappearance of Na^{24} from the plasma. The double component can best be explained by the circumstance that the extracellular sodium is in active interchange with two other body compartments at two different rates. The first compartment includes a significant proportion of the body cells which are readily accessible to the sodium ion and therefore in rapid interchange with the extracellular sodium;³ the second compartment consists of bone and the larger proportion of body cells (relatively impermeable to sodium), which exchange with extracellular sodium at a very slow rate.

The intracellular sodium and potassium concentrations recorded, respectively 35 and 115 mEq/l., do not imply identical concentrations in all tissues; rather they represent average values. It is probably of no significance that their sum is approximately the same as the sum of these cation concentrations in the extracellular compartment. Some tissues contain considerable quantities of magnesium (2), and in any case the activity coefficient of univalent base in tissues is unknown and possibly different from that of plasma.

³ The one-half hour sodium space, which is essentially a manifestation of the rapid component of sodium exchange, is significantly larger than the true extracellular space (table 3).

The average intracellular sodium concentration may require some correction because of excess sodium in bone. The nature of bone sodium is not known. Gabriel was of the opinion that it was present as an insoluble complex salt (21). It has been calculated that excess bone sodium represents 18 per cent of injected Na^{24} (22). Gellhorn and his co-workers (20) have shown that the specific activity of bone tissue increases about 75 per cent during the interval between 2 and 24 hours following the injection of the isotope. The 3-hour sodium measurement used here consequently does not include the total bone sodium. Kaltreider *et al.* (22) have corrected their sodium space for bone sodium and have found an average of 28 per cent of total body weight in the dog as compared with 30 per cent in our uncorrected data. The bone correction will then decrease our calculated intracellular sodium concentration by at most 4 to 5 mEq/l.

The sodium normally present in the red cell of the dog (23) cannot account for the difference between the inulin space and the sodium space since it represents only about 8 per cent of the total sodium.

TABLE 3. VOLUMES OF DISTRIBUTION (PERCENTAGE OF BODY WEIGHT)

DOG	INULIN	SODIUM ²⁴		
		0.5 hr.	3 hr.	24 hr.
A	19.0	23.3	28.1	30.6
G	19.3	24.8	29.0	31.0
H	20.5	25.2	30.2	31.8

Moore (13) has made similar intracellular measurements in man comparing Na^{24} and K^{42} with thiocyanate and D_2O spaces. The values reported are approximately 35 per cent higher than those presented here. There is, however, adequate evidence that thiocyanate space is greater than extracellular space (10, 24-26); consequently any calculation of intracellular water based upon the difference between D_2O and thiocyanate space will be significantly decreased and the apparent concentration of intracellular ions proportionately increased.

If the data presented by Harrison, Darrow and Yannet (2) are corrected by replacing their extracellular fluid measurement by chloride with our figure for the inulin space, namely, 19.4 per cent, the potassium concentration of 140 mEq/l. of cell water which they have reported will be diluted to 116 mEq/l., which figure is in agreement with the value shown in table 1. The intracellular cation concentrations here reported are in close agreement with direct measurements made by Peters on human red cells (27).

The fact that, if appropriate correction is made for renal delay time, the specific activity of urine after the injection of Na^{24} is identical with that of the plasma, warrants the use of urine for the indirect measurement of the plasma specific activity after equilibrium has been reached.

SUMMARY

Intracellular sodium and potassium concentrations in the normal dog have been determined by the simultaneous use of Na^{24} , K^{42} , D_2O , and inulin. The average values

thus determined are 115 and 35 mEq/l. of cell water for potassium and sodium, respectively. The factors which determine the rate of disappearance of Na^{24} and K^{42} from the plasma have been discussed.

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ELECTROPHORETIC STUDY OF PLASMA PROTEINS FOLLOWING HEPATECTOMY IN DOGS

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BOTH chemical and electrophoretic analysis of plasma proteins of patients show that change from the normal occurs in the presence of liver disease. But it is not clear whether the disturbance of the normal pattern is due to the new formation of abnormal proteins, to the lack of formation of particular proteins, or both. To aid in answering the question of protein formation, we have observed the effect removal of the liver might have. While this operation removes the source of most of the plasma proteins, it has the serious disadvantage that the experimental animals seldom live more than 18 hours. Almost to the end, however, they appeared to be in good condition.

Monroe and Avery (1) also have studied this problem, using the electrophoretic technique. They found no significant change up to seven hours after hepatectomy. It seemed desirable to supplement this work by extending the time of study after operation as long as it was possible to keep the animals alive. Further since we have found relatively wide variations in the plasma protein pattern of normal dogs, comparison of samples after hepatectomy were always made with control samples from the same animal.

METHODS

Blood samples were taken from normal, adult dogs in a post-absorptive state before hepatectomy and at frequent intervals thereafter. Seven dogs were studied. Hepatectomy was performed, using the one-stage technic of Firor and Stinson (2). Ether anesthesia was employed for the operation only. During the operation, approximately 125 ml. normal dog's blood was infused. The blood glucose level was maintained by injecting 50 per cent glucose intravenously once an hour. One-half ml. per kg. per hour was given for the first 4 injections. After 4 hours, 1.0 ml. per kg. was used, as it was found in earlier studies that hypoglycemic reactions frequently occurred when the smaller dose was employed. The animals at the time of sampling were active and showed no evidence of hypoglycemia except in the occasional instances noted in the text. The blood samples were taken immediately before the injection, oxalated and the plasma separated.

Electrophoretic studies were carried out using the Tiselius technic as modified by Longworth (3). Phosphate buffer, pH 7.8, ionic strength 0.16 μ , was employed. Total protein was determined by the Pregl modification of the micro-Kjeldahl method. Hemoglobin was determined by the Haden-Hauser method (4).

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RESULTS

The plasma protein pattern of normal dogs shows relatively wide variation (table 1). Because of this variability changes which may occur as a result of hepatectomy can be judged, therefore, only by comparing the pre- and post-hepatectomy

TABLE I
TOTAL PROTEIN CONCENTRATION AND FRACTIONS (ELECTROPHORETIC TECHNIC) OF DOG
PLASMA BEFORE AND AFTER HEPATECTOMY

DOG NO.	HOURS AFTER HEPA- TEC- TOMY	VOLUME PACKED RBC/100 ML. OR HB. GM/ 100 ML.	T. P. GM/100 ML.	ALBU- MIN % OF TOTAL PROTEIN AREA	GLOBULINS: % OF TOTAL PROTEIN AREA					ALBUMIN	MOBILITY: $\mu \times 10^5$ CM. $^{-1}$ SEC. $^{-1}$ VOLT $^{-1}$				
											Globulins				
					α_1	α_2	β	$\phi + \gamma_1$	γ_2		α_1	α_2	β	$\phi + \gamma_1$	γ_2
1407	0	59	5.02	61.4	4.2	3.2	14.5	7.4	9.3	7.1	5.6	4.9	4.0	3.0	1.4
	2	65	4.69	53.6	7.8	5.1	17.4	6.7	9.4	7.0	5.6	4.7	3.8	2.8	1.7
1465	0	14 gm.	6.85	50.9	6.1	4.2	17.2	10.8	10.8	6.4	5.3	4.6	3.5	2.4	1.5
	4	14	5.60	45.9	6.4	4.3	17.7	14.1	11.6	6.1	4.8	4.2	3.3	2.0	0.9
	7		5.55	44.3	7.6	5.6	15.9	13.9	12.7	6.3	5.3	4.5	3.2	2.3	1.4
	9	16	5.63	46.8	5.5	6.0	15.9	13.2	12.6	6.3	5.1	4.3	3.2	2.3	1.1
	10	18	5.51	46.1	4.0	5.9	18.4	14.7	10.9	6.3	5.0	4.6	3.4	2.2	1.2
1482	0	13	6.81	50.6	5.2	4.6	19.2	12.2	8.2	6.2	4.9	4.4	3.3	2.4	1.1
	3½	14.5	7.14	45.5	8.0	5.3	15.9	16.4	8.9	6.2	4.9	4.4	3.4	2.2	1.0
	7½	15.5	6.22	45.5	7.7	3.8	18.7	13.0	11.3	6.5	5.0	4.6	3.6	2.3	1.2
1508	0	15.0	5.84	42.3	7.3	5.4	20.6	14.9	9.5	6.2	5.0	4.4	3.2	2.0	1.1
	5	16.5	7.05	50.2	5.9	3.9	21.8	10.6	7.5	6.4	5.1	4.4	3.4	2.4	1.4
	8½	17.5	6.97	50.0	5.3	5.3	19.3	12.4	7.7	6.1	4.8	4.0	2.9	2.0	1.1
	9½	18.5	6.05	46.7	7.6	5.8	18.2	12.6	9.1	6.2	4.9	4.2	3.1	2.1	1.1
	10½	19. +	6.72	41.9	6.6	5.8	17.5	10.5	4.7	6.1	4.8	4.3	3.1	2.0	1.0
	11½³	21	4.60	46.2	6.8	5.4	18.2	11.6	11.8	6.1	4.9	4.3	3.1	2.1	1.1
1628	0	14	6.09	39.5	10.6	7.7	21.8	14.5	5.9	6.6	5.3	4.5	3.4	2.2	0.9
	3		5.87	37.3	9.5	11.0	18.2	16.2	7.8	6.4	5.5	4.7	3.8	2.4	0.8
	6		5.93	45.3	10.6	8.9	17.3	12.0	5.9	6.3	5.2	4.3	3.5	2.2	0.8
	8	15	5.78	48.9	7.8	8.5	14.5	14.8	5.5	6.8	5.6	4.7	3.6	2.4	0.9
	10	15	6.44												
	12	15.5	5.72	47.0	10.0	5.8	19.3	12.8	5.1	6.6	5.4	4.3	3.5	2.1	0.9
	14	16	5.83	46.8	7.9	5.9	20.0	11.3	8.1	6.7	5.4	4.5	3.6	2.4	0.9
	16	17	5.59	48.2	10.6	8.9	16.4	9.7	6.2	6.7	5.6	4.6	3.5	2.5	0.9
1517	0	13.5	6.35	44.0	8.5	4.9	21.3	16.9	4.4	6.7	4.8	4.1	3.0	1.8	1.0
	3	14.5	6.47	43.0	9.2	7.3	21.3	16.2	3.0	7.0	5.4	4.7	3.3	2.4	1.8
	5	14.0	5.80	46.1	7.7	7.7	15.0	20.1	3.4						
	7½	12.0	5.60	47.5	5.9	6.9	15.2	18.6	5.9	6.9	5.3	4.3	3.5	2.4	1.6
	9	13.0	5.48	47.0	5.9	4.9	20.6	16.9	4.9						
	12	13.0	5.85	47.3	6.3	5.2	21.3	15.5	4.4	6.8	5.3	4.5	3.2	2.3	1.6
1579	0	15.0	6.64	42.4	7.9	7.3	13.7	15.9	12.8	6.1	5.0	4.2	3.2	2.0	0.7
	3	15.0	6.92	42.0	5.7	6.0	18.5	14.0	13.8	6.2	5.1	4.3	3.3	2.1	0.8
	6	16.5	6.55	42.5	7.8	5.3	18.9	12.6	13.9	6.5	5.3	4.6	3.4	2.2	1.0
	8	16.0	6.34	44.4	6.5	4.9	20.5	10.4	13.3	6.3	5.6	4.6	3.3	2.0	0.7
	10	17.0	6.59	44.2	5.7	3.2	20.5	13.6	12.8	6.2	5.2	4.6	3.4	2.2	0.7
	12½	17.0	6.70	44.5	5.0	5.0	22.0	9.9	13.6	6.2	5.2	4.6	3.4	2.3	1.0
	13½	16.5	6.97	43.0	4.2	5.2	20.2	13.7	13.7	6.3	5.3	4.7	3.5	2.3	1.0

¹ In hypoglycemic shock. Blood glucose 18 mg/100 ml. Double usual amount glucose given. ² Active and alert.
³ Drowsy, not improved following glucose. ⁴ Comatose.

pattern. When this was done, it was found that hepatectomy produced small changes in the pattern of the 7 dogs studied. The shifts were not always in the same fraction, nor were they always in the same direction (table 1).

The total plasma protein decreased in 5 of the dogs (1517, 1407, 1465, 1482,

1628) despite the fact that hemoconcentration had occurred, as judged by increased hematocrit and hemoglobin concentrations. The time at which the decrease occurred varied greatly, being 5, 2, 4, $7\frac{1}{2}$ and 16 hours, respectively, after hepatectomy. Three of the five showed a decrease in the total albumin concentration and in the relative percentage of albumin. Four dogs showed an increase in the relative percentage of albumin and no change or a slight increase in the total concentration. The non-protein nitrogen was essentially unchanged.

The changes in the concentration of the globulin fractions were also variable. While 5 of the dogs showed a decrease in β -globulin concentration, 8 to 10 hours after hepatectomy, the sixth, (1579) studied at this time showed a marked increase.

In dog's serum, electrophoresis separates a peak γ_1 with the same mobility as the fraction in plasma which contains fibrinogen. Any change in the ϕ -globulin (more correctly termed $\gamma_1 + \phi$) concentration of plasma does not necessarily indicate a change in fibrinogen concentration. Relatively small changes were observed in the ϕ -globulin concentration within 12 hours after hepatectomy, the greatest decrease being observed in the dog with longest survival (Dog 1628, 16 hour post-hepatectomy). The blood still clotted rapidly and showed normal clot retraction.

The γ_2 -globulin concentration changed little after hepatectomy. Even when the total plasma protein showed marked decrease (Dogs 1465 and 1482) its level was maintained.

There was no significant alteration in the mobility of the various protein fractions. Neither was there any skewing of the peaks, nor any abnormal spiking indicative of the presence of abnormal or modified proteins in the plasma.

The dogs were active and alert at the time of taking the blood samples, with 3 exceptions. Eight hours after hepatectomy dog 1508 was totally unresponsive, but quickly improved after intravenous glucose was given. The blood sugar at the time of collapse was 18 mg. per 100 ml. Dog 1579 was drowsy and unable to stand 12 hours after hepatectomy and was unconscious an hour later. Glucose did not increase his responsiveness. Most of the dogs passed from an active, alert condition into collapse and death, frequently within periods of a quarter to a half hour.

DISCUSSION

The changes which occur in the plasma protein pattern of dogs within 16 hours after hepatectomy are small. This indicates that, in the absence of the liver, both the concentration and normal pattern of the circulatory proteins are maintained for a period of hours.

Berryman, Bollmann and Mann (5) using the Howe precipitation technique, noted that the initial alterations during the first 6 hours after hepatectomy consisted in increase of pseudoglobulin, irregular decrease in fibrinogen, euglobulin, total protein and albumin. There were only slight changes during survival periods of 14 to 30 hours. They believed that the initial changes could be explained by factors other than absence of the liver and stated, "It would appear that in the absence of the liver the loss of protein from the plasma is too small to be measured by our methods within periods up to 30 hours after the removal of the liver." Our

results obtained by the electrophoretic technic are in complete agreement with this view.

Deutsch (6) has concentrated a fraction, γ_1 -globulin from normal human plasma with mobility similar to that of fibrinogen and which is rich in antibodies. It is present in normal plasma to the amount of about 3 per cent and is usually estimated as part of the fibrinogen. In human serum, the γ_1 -peak is small and not clearly defined. Dog serum, however, normally has a relatively large concentration of this component and shows a large, well defined peak. Thus, the patterns of dog plasma and serum when phosphate buffer, pH 7.8 is employed, both show 6 peaks designated for serum as albumin, α_1 , α_2 , β_1 , γ_1 and γ_2 -globulin, in order of decreasing mobility, and for plasma, as albumin, α_1 , α_2 , β , ϕ , and γ_2 -globulin. In the plasma pattern, the ϕ peak is the γ_1 -globulin of serum plus fibrinogen. Because of the relatively large amount of γ_1 -globulin, usually observed in dog's serum, the ϕ -peak of plasma would be more correctly designated as γ_1 and ϕ .

SUMMARY AND CONCLUSIONS

The plasma protein pattern (Tiselius electrophoretic technic) of 7 dogs studied at frequent intervals for periods up to 16 hours after hepatectomy showed only slight changes from the prehepatectomy values. A small fall in the total plasma protein, despite evidence of hemoconcentration, was usually observed. A decrease in the β -globulin fraction usually accounted for part of this fall. The relative mobility and contour of the various protein peaks remained unaltered.

Hepatectomy in dogs surviving up to 16 hours in good condition produces insignificant changes either in the quality or quantity of plasma proteins as measured by the Tiselius electrophoretic method.

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EFFECTS OF 18,000 FEET SIMULATED ALTITUDE ON THE MYOGLOBIN CONTENT OF DOGS

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MYOGLOBIN is a heme-containing pigment of the muscles which resembles hemoglobin in its ability to bind and release oxygen. Whether this characteristic serves animals in acclimatization to life at high altitude is a question of considerable interest. Hurtado *et al.* (1) found that dogs living at high altitude have 66 to 70 per cent more myoglobin in their skeletal muscles than dogs living at sea level. These results indicate that the myoglobin concentrations in the muscles parallel the hemoglobin concentrations in the blood when animals are acclimatized to the reduced amount of oxygen of low atmospheric pressures. Also, they suggest that myoglobin plays an important rôle in the adjustment of muscular metabolism to hypoxic conditions (2).

The work described in this paper was undertaken to find out if the concentration of myoglobin in the muscles increases as a consequence of discontinuous exposure to low pressure as has been found during prolonged continuous exposure (1) and as the hemoglobin does as a consequence of either discontinuous or continuous exposure to low pressure.

METHODS

Dogs and Their Exposure. Seven dogs (3 ♂♂ and 4 ♀♀), whose weights ranged from 5.0 to 8.4 kg. and which had lived in the laboratory long enough to be well adjusted to their routine and food, were selected for the study. The left anterior trapezius muscle was removed from each while heavily anesthetized. The muscle was analyzed for its myoglobin content per gram of wet tissue. After the incisions were completely healed (the dogs showed no effects of losing the muscle), the dogs were exposed to 18,000 ft. simulated altitude (382 mm. Hg) 6 hours per day, 6 days per week for a period of 5 to 6 months.

Before and during the exposure period the weights and the hematocrit and hemoglobin values were determined weekly. The hemoglobin values were determined by measuring the optical density at 541, 560 and 577 mμ of hemolyzed samples of blood diluted 1:250. The densities were multiplied by appropriate constants to obtain the grams-per cent hemoglobin. No special care was taken to control the diet except to offer kennel rations supplemented with a generous portion of horse meat and a pan of milk daily. At the end of the exposure period, the dogs were killed by administering deep anesthesia followed by exsanguination. Samples of the right anterior trapezius, gastrocnemius, biceps femoris muscles, diaphragm and heart were removed for analysis.

Since samples of gastrocnemius, biceps femoris, diaphragm and heart were not analyzed before the exposure, samples were taken from 6 unexposed dogs for control analyses. Of the latter dogs 3 (nos. 8, 9 and 10) had lived in the laboratory 7 months before death and three (nos. 11, 12 and 13) had lived there for several years. The latter had served as sources of blood for other experiments.

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Analyses for Myoglobin. The analyses for myoglobin were made essentially as in a study of the effects of altitude on the myoglobin content of rats (3, 4); however, parts of the procedure have been modified. The details of the procedure as done in these experiments are as follows: The muscles were kept frozen (not more than 3 days) until analyses could be done. Then a sample was carefully dissected free of fat and fascia insofar as possible. About 3 grams were then put into dry ice. After freezing, the meat was pulverized at the temperature of dry ice in a specially designed mortar and pestle (5). A carefully weighed quantity of the pulverized meat was then washed into a homogenizer tube with a carefully measured volume of $M/40$ phosphate buffer, pH 5.9. One to 1.25 grams of tissue were used with 20 ml. of buffer per gram of tissue. The tissue was then thoroughly homogenized with a stainless steel homogenizer and centrifuged at 1000 g for 10 minutes. The supernatant was heated to 56° . As soon as discrete, coagulated particles appeared, the extracts were cooled rapidly, centrifuged at 1000 g for 10 minutes and filtered. The supernatant was a clear red solution.

The filtrate was adjusted to pH 6.5 with solid K_2HPO_4 and then gassed with CO in a rotating tonometer (6). To insure complete conversion to the carbonyl derivatives a trace of $Na_2S_2O_4$ was added to the solution after 10 minutes of gassing. The solutions were gassed two more minutes and transferred anaerobically to absorption cells and the cells stoppered. The density of the solutions was ascertained at 538 and 568 $m\mu$ on a Beckman quartz spectrophotometer.

The densities thus obtained were used to ascertain the concentrations of myoglobin by substituting in the Vierordt equation (7) as follows:

$$C_{mb} = \frac{d_{568} \times \epsilon_{538}^{Hb} - d_{538} \times \epsilon_{568}^{Hb}}{\epsilon_{568}^{Mb} \times \epsilon_{538}^{Hb} - \epsilon_{538}^{Mb} \times \epsilon_{568}^{Hb}}$$

where: C_{mb} = molar concentration of myoglobin, ϵ_{568}^{Hb} and ϵ_{538}^{Hb} = molar extinction coefficients for carbonylhemoglobin at 568 $m\mu$ and 538 $m\mu$, respectively, both of which are 14.7×10^3 . ϵ_{568}^{Mb} and ϵ_{538}^{Mb} = molar extinction coefficients for carbonylmyoglobin at 568 $m\mu$ (11.8×10^3) (6) and 538 $m\mu$ (14.7×10^3) (6), respectively.

The amount of myoglobin per gram of the pulverized muscle is calculated by substituting the molar concentration (M) in the following formula:

$$Mg.Mb/gram\ muscle = \frac{M \times M.W. \times Vol.\ of\ extracting\ fluid}{Wt.}$$

where $M.W.$ = molecular weight of myoglobin (17,300) as determined in previous work (8). $Vol.\ of\ extracting\ fluid$ = volume of the phosphate buffer added plus 0.75 of the weight in grams of the pulverized muscle. $Wt.$ = grams of the pulverized muscle.

Samples of pulverized muscle were analyzed in duplicate. If the amounts of myoglobin in the two samples did not agree within 5 per cent another pair of samples was analyzed.

Tests of Analytical Procedure. During the preliminary work of developing this procedure, a known quantity of dog myoglobin, in a solution 75 per cent pure, was added to the one half of a muscle suspension after homogenization. The concentration

of myoglobin in the supplemented portion was exactly the total of that in the non-supplemented portion plus that added. Another test consisted of analyzing successively 9 samples of one pulverized portion of heart. The myoglobin contents of the 9 samples averaged 3.60 mg.Mb/gm. of muscle with a $\sigma = 0.28$ and a coefficient of variation = 8 per cent.

RESULTS

Changes in Hemoglobin Concentration. The exposed dogs were vigorous and maintained a healthy appearance throughout the months of exposures. Each dog gained weight during the exposures, the gains ranging from 0.2 to 1.5 kg. (table 1). The hematocrit and hemoglobin values rose sharply during the first weeks of exposures. In 5 dogs the values continued to rise during the remainder of the period, but less

TABLE 1. HEMATOCRIT AND HEMOGLOBIN VALUES AND WEIGHTS OF DOGS DISCONTINUOUSLY EXPOSED TO 18,000 FT. SIMULATED ALTITUDE, AND OF UNEXPOSED DOGS

Dog no.	EXPOSED DOGS						UNEXPOSED DOGS			
	Hematocrit Value		Gm. Hb/100 ml. blood		Weight in kg.		Dog No.	Hematocrit value	Gm. Hb/100 ml. blood	Weight in kg.
	Before exposure	After exposure	Before exposure	After exposure	Before exposure	After exposure				
1	56	78	18.3	23.6	7.9	8.7	8	56	18.0	8.1
2	51	60	16.5	20.1	7.7	7.9	9	58	20.8	18.6
3	64	74	21.1	26.4	8.4	9.0	10	58	20.4	9.6
4	51	64	17.8	22.1	5.9	7.1	11	58	22.9	15.3
5	54	80	17.7	25.7	6.1	7.6	12	61	21.9	11.3
6	55	70	18.6	22.6	5.0	6.5	13	56	20.9	10.4
7	42	68	12.8	21.8	5.8	6.2				
Average.....	53	71	17.5	23.1				58	20.8	

sharply. In the other 2 dogs the values decreased after the initial increase; however, during the final month of exposure they regained values equal to those after one month of exposure. Table 1 shows that at the terminus of the exposures each of the dogs had hematocrit and hemoglobin values considerably greater than they had before exposure. They also had an average value greater than that of the unexposed dogs at the time of their death.

Changes in Myoglobin Concentration. The effect of the discontinuous exposure on the myoglobin content of the muscles is well shown in the experiments in which the dogs were their own controls. In 5 of these dogs the right anterior trapezius muscle contained less myoglobin after the exposures than the muscle from the opposite side contained before, and in 2 of the dogs it contained more after the exposures than the opposite before (table 2). The average value, however, is less in the muscles analyzed after the exposures (4.07 mg/gm. muscle) than in the muscles analyzed before (4.33 mg/gm. muscle).

The results of the analyses of the 5 muscles, for which controls were taken from

dogs never exposed, confirm those of the trapezius. It will be seen in table 3 that the average value for the myoglobin content in the trapezius, biceps femoris, gastrocnemius, heart and two regions of the diaphragm was less in the 7 dogs which were exposed to simulated altitude than in the 6 dogs which were never exposed.

These results indicate that discontinuous exposure of dogs confined to the laboratory to simulated altitude causes a decrease in the myoglobin content of their muscles, while simultaneously it causes an increase in the hemoglobin content of the blood.

DISCUSSION

The significance of the differences mentioned above bears consideration. It will be seen in tables 2 and 3 that the standard error of each difference is large and that the probability that these differences occurred by chance is high. The probability values (P) in the skeletal muscles range from 0.05-0.1 to 0.2-0.3. In the heart P is

TABLE 2. MYOGLOBIN CONTENT OF TRAPEZIUS MUSCLE OF DOGS BEFORE AND AFTER 5 TO 6 MONTHS OF DISCONTINUOUS EXPOSURE TO 18,000 FT. SIMULATED ALTITUDE

DOG NO.	MG. MB/GRAM WET MUSCLE		DOG NO.	MG. MB/GRAM WET MUSCLE	
	Before exposure, left trapezius	After exposure, right trapezius		Before exposure, left trapezius	After exposure, right trapezius
1	3.27	2.79	5	4.42	4.67
2	4.69	4.23	6	5.07	3.73
3	5.20	4.70	7	3.77	4.20
4	4.63	4.17			
Average				4.44	4.07

Mean difference = 0.36. Standard error of mean difference = 0.568. $P = 0.1 - 0.2$.

0.4-0.5. Obviously, a hard and fast conclusion that daily exposures of 6 hours to simulated altitude cause a decrease in myoglobin cannot be drawn from these experiments. This is especially true in the heart which seems to have reacted differently from skeletal muscle. It is strongly indicated, however, that such exposure does not cause an increase in myoglobin as found by Hurtado *et al.* (1) in dogs living continuously at high altitude. Also, the results reported above agree with those found in earlier studies on the effects of simulated altitude on the myoglobin content of rats (3, 4).

The fact that the Andean dogs were found to possess more myoglobin than the dogs in Lima has led to the hypothesis that myoglobin possibly plays a rôle in acclimatization to life at high altitude similar to that of hemoglobin, but at the tissue level (2). In the vascular system hypoxemia plays a prominent rôle as a stimulus to the production of hemoglobin, so, by inference, tissue hypoxia would stimulate myoglobin production. Hurtado, Merino and Delgado (2) report average arterial oxygen saturations in two groups of Andean natives as low as 81.4 and 87.6 per cent. Such low hemoglobin saturations would probably result in tissue hypoxia which, if hypoxia is a stimulus of the myoglobin producing mechanism, could well produce higher myoglobin values.

Arterial oxygen saturations were not measured in these experiments, but it has been shown (9) that dogs after short exposure (16 minutes) to 18,500 ft. simulated altitude had arterial oxygen saturations of about 62 per cent. In other exposures (9) at 20,000 ft. (19 to 43 minutes) 5 dogs had saturations ranging from 40 to 54 per cent. The degree of hypoxemia produced in these experiments for 6 hours per day by

TABLE 3. EFFECT OF DISCONTINUOUS EXPOSURE TO 18,000 FT. SIMULATED ALTITUDE ON THE MYOGLOBIN CONTENT OF 5 MUSCLES OF DOGS

DOG NO.	TRAPEZIUS	BICEPS FEMORIS	GASTROCNEMIUS	HEART	DIAPHRAGM	
					Costal	Lumbar
<i>Exposed Dogs</i>						
		<i>Mg. Mb/gm. wet muscle</i>				
1	2.79	5.75	6.00	3.44	5.25	
2	4.23	5.66	5.79	3.56	5.35	5.14
3	4.70	5.95	5.94	3.00	7.33	
4	4.17	5.77	7.16	4.00	6.58	6.04
5	4.67	6.45	7.42	4.49	7.33	7.65
6	3.73	5.30	5.30	3.73	4.98	
7	4.20	5.74	6.54	3.82	5.21	5.09
Average.....	4.07	5.80	6.31	3.72	6.00	5.98
<i>Unexposed Dogs</i>						
		<i>Mg. Mb/ gm. wet muscle</i>				
8	3.13	5.34	6.57	3.49	6.30	6.10
9	4.33	6.53	7.07	4.04	6.40	6.49
10	3.98	5.97	6.61	3.78	6.02	6.50
11	6.40	7.90	7.18	3.95	7.58	7.23
12	5.52	7.08	7.32	3.85	6.48	7.42
13	4.56	6.10	5.67	4.11	6.78	8.19
Average.....	4.65	6.49	6.74	3.87	6.59	6.98
Difference between averages.....	0.58	0.69	0.43	0.15	0.59	1.00
S ¹	0.92	0.37	0.34	0.21	0.48	0.58
P.....	0.2-0.3	0.05-0.1	0.2-0.3	0.4-0.5	0.2-0.3	0.1-0.2

¹ *S* = standard error of differences.

18,000 ft. simulated altitude, however, did not stimulate the myoglobin-producing mechanism.

There are several possible explanations of the disagreement between the results found in this laboratory on the dog and rat (3, 4) and those found by Hurtado *et al.* (1) in the Andes on the dog. One is the vast difference in the amount of exposure to low atmospheric pressure. Another is the difference in methods of analysis. Hurtado *et al.* used Whipple's method (10) in which simultaneous bleeding and perfusion were

performed to remove hemoglobin and the extracted myoglobin was estimated as acid hematin by colorimetric comparison with hemoglobin hematin standards. It is doubtful, however, that differences in methods account for the lack of agreement in the two experiments.

The influence of exercise in the production of myoglobin in these experiments bears consideration in view of the results of Whipple and his colleagues (10, 11). They found that physical condition and exercise are very influential in determining the myoglobin content of dog and human muscle. The influence of exercise in these experiments was nil. Between exposures the dogs were confined in cages. During exposures they were leashed to the sides of the chamber and had about the same freedom as in the cages. The controls were confined to cages throughout their stay in the animal quarters. Study of the results of Hurtado *et al.* suggests that the amount of exercise during the weeks preceding analysis influenced the quantity of myoglobin found in the Andean dogs.¹ These considerations emphasize the desirability of studying the effects of exercise superimposed upon hypoxia on the myoglobin content. This study, together with the results of the rat (3, 4), indicates that the production of myoglobin is not stimulated by the hypoxia caused by 6 hours' daily exposure to 18,000 ft.

SUMMARY

Two experiments were made to study the effect of 6 hours' daily exposures to simulated altitude on the myoglobin content of muscle in 7 dogs. In one experiment the anterior trapezius of one side was analyzed before the initial exposure and the muscle of the other side analyzed after the final exposure. In the other experiment the myoglobin content of 5 muscles in the exposed dogs was compared with the content of the same muscles in 6 unexposed dogs. In all exposed dogs the hemoglobin content increased markedly during the exposure but the average myoglobin content of the trapezius decreased slightly. Also, the myoglobin content of the other 5 muscles was less in the exposed dogs than in the unexposed.

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¹ The dogs used at sea level in the Peruvian experiment were reared in Lima and kept for 8 weeks on a uniform diet and moderate activity before analysis. Of the 7 dogs used at high altitude 4 were kept for 8 weeks on a uniform diet before analysis, and 3 were analyzed immediately after acquisition. The results (1) show that the quantity of myoglobin was less in the muscles of the 4 dogs which were kept in the laboratory for 8 weeks than in the 3 which were analyzed immediately after acquisition.

RELATION OF THIOLS TO HEAVY METAL INHIBITION¹

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RECENT studies of the mechanisms of heavy metal inhibition of metabolism in the synovialis have demonstrated the stabilizing effect of glutathione (1). Heavy metal ions at concentrations which normally produce high positive membrane potentials have been shown to be relatively ineffective in the presence of glutathione at approximately equivalent concentrations. It has accordingly been suggested that the stabilization of membrane potentials normally observed at low concentrations of metal results from reduction of the ionic oxidant by sulfhydryl groups. In order to determine the metabolic effects of the interaction between thiols and heavy metals in tissues, the investigation has been continued, with particular reference to copper and iron.

EXPERIMENTAL RESULTS

The method of determination of the synovial membrane potentials in dogs has been previously described (2, 3). In present investigation, saturated KCl^- calomel electrodes have been employed, and liquid junctions have been made in the manner previously reported.

The systems studied include combinations of copper or iron with various thiols. The latter group includes substances which are normal constituents of tissues, glutathione, cysteine and methionine as well as such compounds as thioglycolate, thiourea, thiouracil and BAL. In addition to the thiols, ascorbic acid, glycine and alanine have been studied as examples respectively of a non-thiol reducing substance and of non-thiol amino acids. The effects of cuprous ions have also been determined.

The interaction between the metal ions and the other agents was determined by following the membrane potential during alternate applications of the metallic ion in isotonic NaCl, followed by thiol or other agent in isotonic NaCl. The usual procedure was to apply a solution containing the heavy metal ion for 90 seconds, followed by a duplicate solution for 90 seconds. During this 3-minute period potentiometer readings were taken every half minute, 4 being obtained for each application. Averages of the 8 readings were computed, indicating the level of the potential during the period. During alternate 3-minute periods duplicate solutions of thiol or other reagents were applied in the same manner. The effects of alternating heavy metal ions and thiols were determined over periods of 30 minutes or more. As control, the same procedure was applied to 5 animals in which the cupric ion was alternated with isotonic NaCl containing no thiol or other reagent.

The experimental results are represented graphically in figures 1 and 2. For the sake of clarity they are presented in the form of individual experiments, each of which is representative of the results obtained from a series of experiments. The potentials

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represent the averages of 8 readings obtained during the application of a given solution for 3 minutes.

In addition to the series of experiments enumerated above, the effects of applied external voltage, ± 1 volt applied for 1 minute in the presence of cupric ions, have been studied. The method of applying external voltage to polarize the membrane has been previously described (2). In the experiments with cupric ions, the potential was first produced by treating for 3 minutes with duplicate solutions of 7.5×10^{-4} M CuCl_2 in isotonic NaCl. Galvanic current was then applied for 1 minute, after which the circuit was broken and the potentials redetermined over a period of about 10

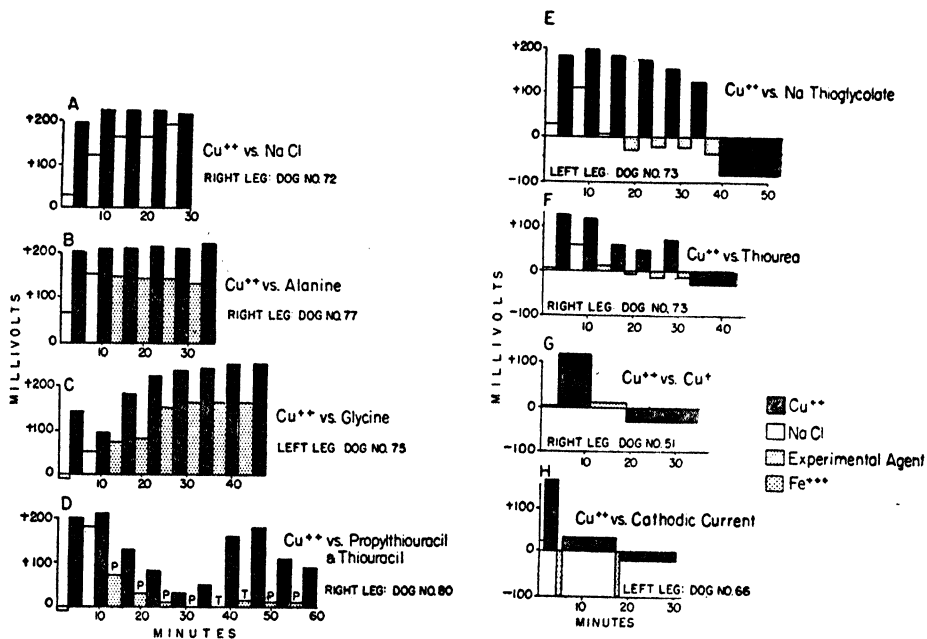


Fig. 1. EFFECTS OF THIOLS and other agents on potentials produced by heavy metals. Ordinate is potential in mv.; abscissa, time in min. Blocks are shaded as follows: cupric ions, diagonal lines; ferric ions, crossed diagonal lines; isotonic NaCl, unshaded. Dotted areas represent experimental agent in each case.

minutes. The current was applied again for 1 minute, and the potentials were re-determined over another 10-minute period. As controls, there have been previously reported determinations of polarization potentials with isotonic NaCl as well as other alkali and alkaline earth salts (2). The results of these experiments have shown that under similar conditions, the polarization potentials approached zero within 2 minutes after breaking the current. The effects of anodic and cathodic currents on cupric ion potentials are included in the results given in table 2; the effect of the cathodic current is shown in figure 1 H.

Data referring to the primary potentials of cupric and ferric ions and to those of the various thiols are given in table 1. These values were obtained in the synovial cavity under conditions in which the tissue had previously been exposed only to

isotonic NaCl. A summary of the effects of experimental agents on the potentials of the heavy metal ions is given in table 2. Those reagents which when alternated with the heavy metal gave results essentially the same as the NaCl control series are included in the first group of table 2. The second group includes those reagents which caused the heavy metal potential to become negative with respect to the original NaCl base line. The third and fourth groups include respectively the reagents which caused the heavy metal potential to become less positive or more positive than the NaCl control series.

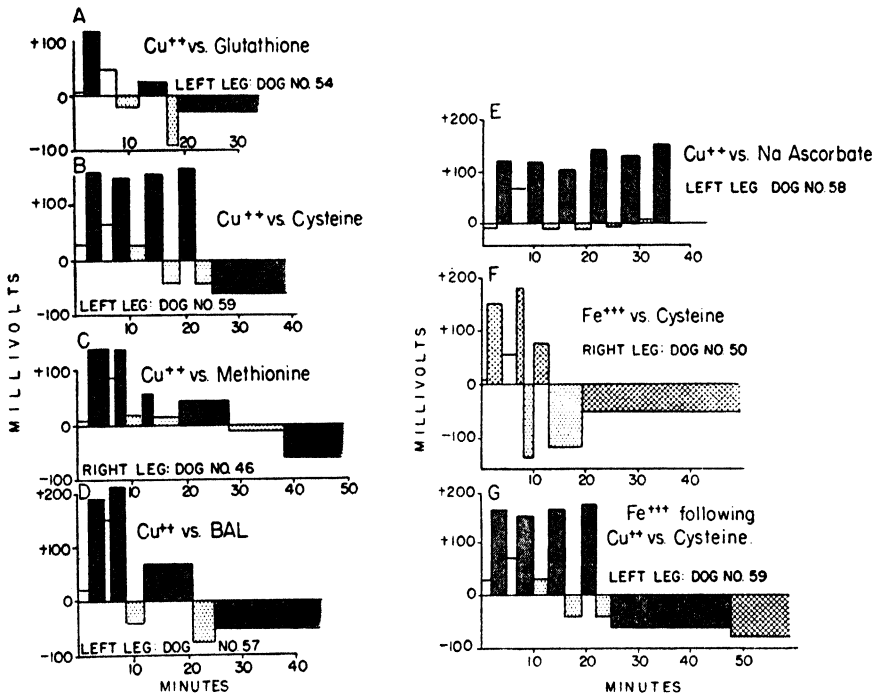


Fig. 2. EFFECTS OF THIOLS and other agents on potentials produced by heavy metals. Explanation as in fig. 1.

It is evident from the qualitative tabulation of results in table 2 that alanine, sodium ascorbate and the anodic current had no significant effect on the copper potentials. Of all the reagents, only glycine tended to increase the potentials higher than the NaCl control level. A typical glycine effect is shown in figure 1 C. Two of the thiols, thiouracil and propylthiouracil, (fig. 1 D), caused the cupric potentials to become less positive than the NaCl controls, but failed to make them more negative than the NaCl base line. Six other reduced thiols, namely glutathione, thiourea, sodium thioglycollate, cysteine, methionine, and BAL caused the cupric potentials to become more negative than the NaCl base line. Cuprous ions and the cathodic current also showed the same effect (fig. 1 G and H). The ferric ion potential, ordinarily between 150 and 200 millivolts positive, was rendered more negative than the

base line by cysteine (fig. 2 F), and by previous production of a negative cupric potential, either by cysteine (fig. 2 G) or by cuprous ions (fig. 2 H).

The results thus clearly differentiate two groups of substances. Reduced thiols either cause the cupric potentials to become negative to the original base line or to approach the base line. The effect is not caused by the amino group, for it is not given by glycine or alanine, nor is it caused by a low redox potential per se, for sodium ascorbate does not show the effect. The results also clearly differentiate the anodic and cathodic currents, the former being ineffective on the cupric potentials, while the latter cause the potentials to become negative.

DISCUSSION

Results to be explained include the following effects: 1) the positive potentials yielded by heavy metal oxidants; 2) those produced by various thiols; 3) the effects of thiols on the heavy metal potential, and 4) the corresponding effect of various

TABLE 1. PRIMARY POTENTIALS OF METALLIC IONS AND THIOLS

REAGENT	CONCENTRATION	MEAN POTENTIAL	REF. ¹	REAGENT	CONCENTRATION	MEAN POTENTIAL	REF. ¹
	mMol/l.	mV.			mMol/l.	mV.	
Fe ⁺⁺⁺	7.5	+149	1	Thiourea	30.0	+220	3
Cu ⁺⁺	0.75	+146	1	Na Thioglycolate	30.0	+170	3
Glutathione	10.0	-10	3	Thiouracil	10.0	+50	3
Cysteine	15.0	-5		2-3 mercapto propanol (BAL)	15.0	-10	
Methionine	15.0	-5					

¹ Values for cysteine, methionine and BAL obtained from data of present investigation.

non thiols. In two previous papers (1, 3) the primary effects of the thiols and heavy metal ions have been interpreted in terms of oxidoreductions in the cytochrome system. The observed potentials in thiol-heavy metal systems are evidently explicable in terms of the same mechanisms.

The thiols are clearly divided into two groups. The first, including cysteine, methionine, glutathione and BAL, produce small negative primary potentials (table 1). This group is most effective in lowering cupric ion potentials to negative values (table 2). The second group, including thiourea, thiouracil, thioglycolic acid and propylthiouracil, produces high positive primary potentials and is less effective in lowering the cupric ion potentials. With the exception of BAL, the first group consists of naturally occurring thiol amino acids and peptides, while the second group consists of thiols known to behave as metabolic inhibitors. The previous interpretation of the positive potentials obtained with thiol inhibitors postulates their oxidation via cytochrome c, the standard redox potential of which is approximately 300 millivolts more positive than that of cytochrome b (4). Accordingly the difference of potential in the cytochrome system is lower when oxidations occur at the higher level. The naturally occurring thiol amino acids and glutathione fail to show this effect,

indicating their tendency to adjust the potential to the normal level. In addition they tend to abolish rapidly the primary cupric ion potentials. This is readily understood if these potentials are produced by the oxidation of lower cytochromes at the cytochrome b level of potential (3). A thiol of the first group, present in excess, is able apparently to reduce the cytochrome in the presence of heavy metal ions. Thiols

TABLE 2. EFFECTS OF REAGENTS ON HEAVY METAL POTENTIALS

AGENT ALTERNATED WITH METALLIC ION	TYPE OF AGENT	ION	CONC. OF AGENT	NO. EXPER.	FIG.
<i>mMol/l.</i>					
<i>I. Systems not Significantly Different from Control Series</i>					
NaCl	Control	Cu ⁺⁺	150.0	5	1A
Na ascorbate	Non-thiol reducing agent	Cu ⁺⁺	15.0	2	2E
DL-alanine	Non-thiol amino acid	Cu ⁺⁺	15.0	2	1B
Anodic current	Potential grad.	Cu ⁺⁺	(1 v.)	4	
<i>II. Systems Producing Potentials Negative to NaCl Base Line</i>					
Glutathione	Thiol tripeptide	Cu ⁺⁺	15.0	4	2A
DL-cysteine	Thiol amino acid	Cu ⁺⁺	15.0	3	2B
DL-methionine	Thiol ester amino acid	Cu ⁺⁺	15.0	4	2C
2-3 mercapto propanol (BAL)	Dithiol	Cu ⁺⁺	15.0	2	2D
Thiourea	Non-amino thiol	Cu ⁺⁺	15.0	2	1F
Na thioglycolate	Non-amino thiol	Cu ⁺⁺	15.0	3	1E
Cuprous ion	Reduced metal ion	Cu ⁺⁺	0.0025	4	1G
Cathodic current	Potential grad.	Cu ⁺⁺	(-1 v.)	4	1H
DL-cysteine		Fe ⁺⁺⁺	15.0	3	2F
Cysteine-Cu ⁺⁺		Fe ⁺⁺⁺	15.0	3	2G
Cu ⁺⁺ vs. Cu ⁺		Fe ⁺⁺⁺	0.0025	3	
<i>III. Systems Producing Potentials Positive to Base Line but Negative to Control</i>					
Thiouracil	Non-amino thiol	Cu ⁺⁺	10.0	6	1D
Propylthiouracil	Non-amino thiol	Cu ⁺⁺	10.0	5	1D
<i>IV. System Producing Potential Higher than NaCl Control</i>					
Glycine	Amino acid	Cu ⁺⁺	15.0	5	1C

Cu⁺⁺ concentration, 0.75 mM; Fe⁺⁺⁺ concentration, 7.5 mM.

of the second group produce similar effects but far less rapidly presumably because as inhibitors they are oxidized by cytochromes of higher potential.

Ascorbic acid, although producing negative primary potentials, is unable to abolish the cupric ion potentials. Apparently its catalytic oxidation by cupric ions occurs predominantly in the liquid phase, by passing the cytochromes.

An applied external cathodic field inhibits the electron current from cytochrome b to cytochrome oxidase and theoretically favors the reduction of the lower cytochromes. Such a field abolishes cupric ion potentials by favoring the reduction of cupric ions rather than oxygen. The mechanism accounts for the effectiveness of the cathodic field in abolishing the potentials and also for the ineffectiveness of the anodic

field. Negative potentials produced by cuprous ions or by the effect of a cathodic field on cupric ions are very difficultly reversed by heavy metal oxidants.

It is concluded that the previous interpretation of the potentials given by thiols and heavy metals adequately accounts for the interactions between the two groups of substances, and also that the interpretation may be broadened to include the effects of external fields.

SUMMARY

The effects of a number of thiols on membrane potentials produced by cupric ions have been observed in the synovialis of dogs. The effects have been compared with those of glycine, alanine and ascorbic acid, as well as with anodic and cathodic currents.

The most effective substances in abolishing the positive potentials of cupric ions were glutathione, cysteine, methionine and BAL. Monothiols containing no ionized amino groups tended to abolish the potentials more slowly than the amino thiols. As a group they produced high positive primary potentials in contrast with glutathione and cysteine. Glycine, alanine, ascorbic acid and anodic current were ineffective in lowering the cupric potentials. Cathodic current or cuprous ions were about equally effective in lowering the cupric potentials.

The effects are interpreted as reduction of cytochrome electron acceptors, counteracting the oxidations caused by cupric ions.

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RELATIONSHIP OF INSULIN HYPOGLYCEMIA TO INTESTINAL SECRETION¹

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INSULIN hypoglycemia is known to stimulate the vagus center. Associated with such stimulation are augmented gastric (1) and pancreatic secretion (2) and changes in gastric (3) and intestinal motility (4). Direct stimulation of the vagus nerve increases the secretion of digestive enzymes by the small intestine (5). In view of the increasing use of vagotomy as treatment for various disorders of the alimentary tract it was considered important to study intestinal secretory activity during vagal stimulation due to insulin hypoglycemia.

METHODS

Two female dogs were prepared with jejunal transplants in which the mesenteric nerves and blood vessels were left intact (6). The animals were maintained on a diet of dog biscuit³. With the aid of soft rubber catheters placed in the fistulas, hourly collections of the secretions were made and the volumes recorded for a period of seven hours. At the end of this time, the pooled collection for the day, solid clumps as well as liquid portion, was homogenized by means of an electric beater.

Enzyme activity was determined by incubating 1.0 ml. of intestinal juice with 25.0 ml. of the appropriate substrate (12% sucrose solution, and 5% peptone⁴ solution buffered to pH 7.8 for sucrase and peptidase respectively) for 16 hours at 37° C. Glass distilled water was used exclusively and 1.0 ml. of boiled secretion was added to the control blanks. Sucrase activity is expressed as mg. of reducing sugar (7) released by 1.0 ml. of secretion. Peptidase activity is expressed as mg. of amino nitrogen as determined by the Sørensen formol titration (8). The activity of 1.0 ml. of secretion multiplied by the seven hour volume of secretion was taken as a measure of the total enzyme output of the jejunal transplant. The diurnal blood sugar pattern was determined at frequent intervals (7, 9). Before the beginning of a collection of juice the fistulas were carefully flushed out with isotonic saline. Collections were made alternately immediately after feeding $\frac{1}{2}$ of the daily ration and after 16 hours of fasting.

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² The data in this paper are taken from a thesis presented by Albert W. Kneller to the Graduate School, University of Rochester in partial fulfillment of the requirements for the degree Master of Science, 1948.

³ Purina Laboratory Chow.

⁴ Difco-Bacto.

Control observations were made on both the fasted and fed animals before and after an approximately equal number of experimental observations during which an attempt was made to maintain the blood sugar concentration just above shock level. This condition was achieved by the subcutaneous injection of appropriate amounts of crystalline and globin insulin. The amount of insulin required was carefully determined for each dog and controlled in every experiment on the basis of hourly blood sugar analyses. In order to avoid possible delayed effects of insulin, a rest period of 9 days was allowed before subsequent control experiments were begun.

The secretory responses of an intestinal fistula vary from day to day over a rather wide range. The first series of experiments were conducted from February to August and it was evident that secretion was diminished during extremes of temperature. In an attempt to determine the influence of temperature, several series of control observations were made in which the animals were not removed from a thermostatically controlled room. Secretory responses were observed at 21 , 26 and $31 \pm 1^\circ \text{C}$. Experiments were begun only after a 7 day period of adaptation to each temperature. Since the maximum response, at controlled temperatures, occurred at 26°C ., this temperature was selected for a repetition of the insulin experiments previously performed at room temperature.

RESULTS

The experimental data obtained at prevailing room temperatures are summarized in table 1. Results obtained at 26°C . are found in table 2. The effects of three environmental temperatures are summarized in table 3.

With a single exception, *dog 1* produced more enzymes at room temperature than at 26°C . This is true also for *dog 2* in 11 of 14 possible similar comparisons. It appears, therefore, that a fluctuating temperature exerts a stimulatory but a quantitatively unpredictable influence upon the intestinal fistulas. It should be emphasized that the extrinsic innervation to the fistulas remained intact. Fistula temperature was never more than 0.6°C . cooler than rectal temperature regardless of room temperature and in most experiments the gradient was about 0.3°C . The rectal and fistula temperature differences were not consistently related to ambient air temperatures.

A comparison of the corresponding values for the fasted versus the fed animals reveals the inhibition, due to feeding, not uncommonly manifested by innervated intestinal fistulas in the absence of direct contact with chyme. The data for the initial and final control periods indicate that the secretory function remains unchanged for at least six months despite the lack of local stimulation from normal intestinal contents.

The following presentation of results is abbreviated by referring specifically to the secretion of sucrase but, with only one exception, the secretion of peptidase followed a parallel course.

It is evident from the data in table 1 that moderate hypoglycemia (blood sugar 25–50 mg.%) in the *fasted* animal leads to a slight increase in secretion of intestinal enzymes. The change is significant only in *dog 2* ($+37\%$; $P=0.004$). On the other hand, if the animals were *fed* just prior to the collection of juice a similar hypoglycemia

resulted in a striking inhibition of sucrase secretion which was 80 per cent for *dog 1* ($P=0.005$) and 50 per cent for *dog 2* ($P=0.045$). The degree of inhibition was computed on the least favorable basis by comparing the lower values in control period 1 with those obtained in hypoglycemia.

During severe hypoglycemia (blood sugar 20-25 mg.%) in the fasting condition the sucrase output of the intestinal fistulas was very much diminished. In *dog 1* the enzyme output was decreased 49 per cent ($P=0.035$) and in *dog 2* it was 29 per cent

TABLE 1. INTESTINAL SECRETION AS AFFECTED BY INSULIN HYPOGLYCEMIA AT ROOM TEMPERATURE
Averages for 7-hour collections of juice

EXPER. CONDITION	NO. EXPERS.	DOG 1			NO. EXPERS.	DOG 2		
		Peptidase (NH ₂ -N)	Sucrase (Sugar)	Volume		Peptidase (NH ₂ -N)	Sucrase (Sugar)	Volume
		mg.	mg.	ml.		mg.	mg.	ml.
<i>Fasted</i>								
Control 1	5	505	10,760	19.5	5	628	11,560	44.8
B.S. ¹ 67-50 mg. %		±52 ²	±1,470	±1.9		±45	±306	±3.5
Insulin hypoglycemia	6	536	11,400	25.0	4	808	15,870	43.9
B.S. 50-25 mg. %		±40	±846	±2.2		±33	±1,070	±4.4
Insulin hypoglycemia	3	292	5,560	16.6	4	508	8,356	42.1
B.S. 25-20 mg. %		±73	±1,480	±2.2		±53	±867	±4.3
Control 2	11	558	10,612	20.2	7	723	11,132	48.1
B.S. 71-67 mg. %		±38	±905	±2.4		±36	±512	±3.5
<i>Fed</i>								
Control 1	6	459	7,060	26.1	5	494	7,420	46.2
B.S. 71-58 mg. %		±50	±821	±1.8		±70	±1,220	±1.8
Insulin hypoglycemia	5	189	1,418	18.4	4	448	3,760	43.0
B.S. 75-36 mg. %		±45	±320	±3.7		±65	±609	±4.5
Insulin hypoglycemia	1	455	8,351	28.8	4	624	10,955	51.0
B.S. 40-25 mg. %						±62	±870	±1.0
Control 2	8	524	9,665	20.6	10	667	11,222	52.6
B.S. 76-60 mg. %		±75	±1,478	±3.1		±30	±1,320	±3.0

¹ Blood sugar.

² Standard error.

($P=0.007$). It was difficult to maintain the severe hypoglycemia in the fed animal, and the blood sugar in these experiments covers the range from 25 to 40 mg. per cent. It is evident from the data in table 1, however, that the lower range of blood sugar concentration resulted in a return of the secretory response to normal.

The data in table 2 indicate that the pattern of reactions of the small intestine to insulin hypoglycemia, first observed under conditions of prevailing temperature, was reproduced qualitatively at constant environmental temperature. Also if the blood sugar concentration of the fed animals was 30 mg. per cent or lower during the entire seven hour collection period, there was a 100 per cent increase ($P=.001$) in secretory activity for *dog 2* and a return to normal for *dog 1*. With the constant tem-

perature condition the agreement between the mean values for control periods 3 and 4 was much improved and the standard errors of the means were generally smaller. It was observed that raising the environmental temperature from 21 to 26° C. served to stimulate the intestinal glands (table 3) but raising the temperature further, to 31° C., had the opposite effect. It should be noted, however, that the absolute values obtained at constant temperature (table 2) are smaller than those obtained at prevailing temperature (table 1).

The changes in volume of intestinal juice appear not to be consistently related either to the output of enzymes or the concentration of blood sugar.

TABLE 2. INTESTINAL SECRETION AS AFFECTED BY INSULIN HYPOGLYCEMIA AT 26°C.
Averages for 7-hour collections of juice

EXPER. CONDITION	NO. EXPER.	DOG 1			NO. EXPER.	DOG 2		
		Peptidase (NH ₂ -N)	Sucrase (Sugar)	Volume		Peptidase (NH ₂ -N)	Sucrase (Sugar)	Volume
		mg.	mg.	ml.		mg.	mg.	ml.
<i>Fasted</i>								
Control 3	6	381	7,390	17.0	6	582	13,130	47.5
B.S. 60-55 mg. %		±11	±778	±2.2		±30	±661	±2.1
Insulin hypoglycemia	2	412	8,290	20.8				
B.S. 37-10 mg. %								
Insulin hypoglycemia	1	244	4,530	10.3	2	337	6,805	40.7
B.S. 30-11 mg. %								
Control 4	3	348	6,900	17.8	2	533	10,780	50.1
B.S. 67-54 mg. %		±8	±145	±1.5				
<i>Fed</i>								
Control 3	4	218	3,470	12.5	5	366	6,040	47.1
B.S. 70-50 mg. %		±17	±338	±1.0		±8	±353	±3.6
Insulin hypoglycemia	1	162	2,820	8.7	1	292	3,760	51.1
B.S. 35-15 mg. %								
Insulin hypoglycemia	1	270	4,090	20.7	2	547	11,259	68.2
B.S. 25-15 mg. %						±48	±350	±3.0
Control 4	3	223	3,890	15.1	2	335	4,870	47.7
B.S. 69-57 mg. %		±18	±150	±1.8		±114	±2,180	±6.5

DISCUSSION

The data obtained from this investigation demonstrate that the small intestine reacts to insulin-induced hypoglycemia in much the same manner as the stomach and pancreas. Measuring simultaneously both gastric secretion and motility in the fasted dog, Necheles *et al.* (3, 10) demonstrated a two-phase response to an insulin injection of 0.65 units per kilogram of body weight. A stimulation of motility and secretion was associated with a moderate insulin reaction, and a depression of gastric motility and secretion with a severe hypoglycemia, i.e. blood sugar below 27 mg. per cent.

Friske and Welin (2) demonstrated that with the increase in gastric secretion

caused by the injection of 'moderate amounts' of insulin, there was a coincident increase in the pancreatic secretion of enzymes. Scott *et al.* (11) measured gastric motility and pancreatic secretion in dogs injected with one unit of insulin per kilogram of body weight. Recalculation of their data to find the total enzyme secretion rate for each half hour period, indicates that they were able to effect an inhibition of pancreatic secretion at very low blood sugar concentrations. Thomas and Crider (12) mentioned that when their fasted dogs were in a convulsive state due to insulin injection the pancreatic secretion was inhibited.

It is evident, therefore, that a moderate hypoglycemia in the fasted animal stimulates the stomach and pancreas and may or may not stimulate the small intestine, and that severe hypoglycemia causes inhibition in all the digestive glands thus far studied.

TABLE 3. RELATIVE VALUES OF INTESTINAL SECRETORY ACTIVITY AT DIFFERENT CONSTANT ENVIRONMENTAL TEMPERATURES. TOTAL AMOUNT OF SECRETION PER DAY, AT ENVIRONMENTAL TEMPERATURE OF 21°C., SET RELATIVELY EQUAL TO 1.00

OBSERVATION	21°C. 3 EXPERIMENTS		26°C. 6 EXPERIMENTS		31°C. 3 EXPERIMENTS	
	Dog 1	Dog 2	Dog 1	Dog 2	Dog 1	Dog 2
<i>Fasted</i>						
Sucrase	1.00	1.00	0.98	1.86	0.90	1.34
Peptidase	1.00	1.00	1.10	1.32	0.80	1.16
Volume	1.00	1.00	1.18	1.00	0.96	1.13
<i>Fed</i>						
Sucrase	1.00	1.00	1.14	1.90	1.10	1.53
Peptidase	1.00	1.00	1.12	1.44	1.05	1.28
Volume	1.00	1.00	0.78	1.16	0.87	1.25

The rapid pancreatic secretion (11) and increased gastric secretion (12) noted for the fasted animal in response to a moderate dose of insulin is abolished by vagotomy. The inhibition of gastric motility which is observed when sufficient insulin is given to a vagotomized animal persists after section of the splanchnics, removal of one adrenal and the medullary portion of the other, and coeliac ganglionectomy (13).

It is very interesting that insulin hypoglycemia should have opposite effects in the fasting and fed animals. The blood sugar in every instance was determined in peripheral venous blood and of course to achieve a comparable depression the fed dog was given a larger dose of insulin. The portal blood sugar in the absorptive state was probably much higher in the fed than in the fasting condition but it is difficult to see how this could account for the difference in secretory response of the intestinal fistula. The only other obvious difference is the presence of more insulin in the fed animal but the possible effect of insulin per se on the intestinal glands remains to be determined.

The adrenal medulla doubtless responds to hypoglycemia in the usual way and an excess of epinephrine or sympathin may account for the inhibition noted under

certain conditions. But here again it is difficult to see why the responses to the same degree of hypoglycemia should be so different in the fasting and post-prandial state unless the sugar concentration in the portal vein is a determining factor.

It is interesting that graded degrees of anoxia will bring about responses which are very similar to those produced by insulin (14-16).

Salle (17) has demonstrated a decrease in gastric secretion of pepsin and rennin by changing the environmental temperature from 26 to 32° C. Environmental temperature appears to be an important factor in gastrointestinal secretory activity, and the control of this variable, and possibly the humidity as well, might lead to more consistent gastrointestinal responses.

SUMMARY

In one fasting dog insulin hypoglycemia, with blood sugar above 25 mg. per cent, stimulated the intestinal glands to an increased output of digestive enzymes. In another animal the change was insignificant. If the blood sugar was reduced below 25 mg. per cent both animals showed marked inhibition of enzyme secretion.

In the fed animals insulin hypoglycemia, with blood sugar above 30 mg. per cent, induced a striking inhibition of enzyme secretion. A post-prandial depression of blood sugar below 30 mg. per cent in one animal resulted in a return to normal, and an increase in the other.

Changes in the volume of intestinal juice were not consistently related to the concentration of blood sugar.

Animals kept successively at 21, 26 and 31°C. gave the greatest secretory response at 26° C.

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EFFECT OF TEMPERATURE UPON THE GLUCOSE TOLERANCE OF THE EVISCERATE RAT

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THE data of these experiments show that the tolerance of the eviscerate rat for intravenously administered glucose is increased as the temperature of the air surrounding the animal is increased.

METHODS

Male rats of the Sprague-Dawley strain were fed Archer Dog Pellets. The two-stage procedure for evisceration has been described (1). When the animals reached a weight of 250 ± 2 grams, they were anesthetized (intraperitoneal injection of 18 mg. of cyclopentenyl-allyl-barbituric acid sodium) and were subjected to the second stage of evisceration. Asepsis was preserved in both stages of the operation. Intravenous injections of solutions containing 0.9 per cent sodium chloride and varying concentrations of glucose (C.P. Dextrose, Merck) with and without regular insulin (Lilly) were made by a continuous injection machine which delivered fluid from each of 6 syringes at the rate of 20 cc. in 24 hours. The glucose load is expressed as milligrams of glucose per 100 grams of rat per hour (mg/100/hr.). The insulin dose was 4 units per 24 hours per rat.

Infusions were made into the saphenous vein of the right hind leg and were started within 5 minutes following the removal of the liver. The animals were secured in a supine position on an animal board and were enclosed in a cabinet which permitted the regulation of temperature within $\pm 0.5^\circ\text{C}$. The analyses of blood glucose were made by the method of Miller and Van Slyke (2). The samples of blood were taken from the jugular vein at the end of 3 hours of infusion.

EXPERIMENTS AND RESULTS

In *experiment 1*, 39 groups of eviscerate rats, having 6 animals per group, were infused with glucose and insulin for 3 hours. Various glucose loads were tested at each temperature until an amount was found which sustained the average level of blood glucose at approximately 100 mg. per cent (its initial level) during the 3 hours of infusion. This glucose load was considered to represent tolerance. Although averages based upon 6 animals are unstable due to individual variability there was a striking correlation between temperature and glucose tolerance. At a temperature of 38°C ., a glucose load of 150/100/hr. was tolerated, whereas at 24°C . a glucose load of 60/100/hr. represented tolerance.

In *experiment 2*, 11 groups of eviscerate rats, having 6 rats per group, were infused with glucose without insulin for 3 hours. Temperatures of 26 and 38°C . were

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tested. At 26° the glucose load of 10/100/hr. best represented tolerance and at 38° the glucose load which best represented tolerance was 22/100/hr.

DISCUSSION

Following evisceration the temperature of the rat falls until it approaches the temperature of the room to within 1 or 2°. The data of these experiments show that as the temperature of the cabinet is increased, there is a corresponding increase in glucose tolerance. Russell and Cappiello (3) have shown a positive correlation between temperature and the level of amino acids into the blood of the eviscerate rat.

TABLE 1. AVERAGE¹ LEVELS OF BLOOD GLUCOSE (MG. %) IN THE EVISCERATE RAT AT THE END OF 3 HOURS OF INFUSION WITH GLUCOSE AND INSULIN

TEMPERATURE OF AIR, °C.	GLUCOSE LOAD, MG/100/HR.											
	160	150	140	130	120	110	100	90	80	70	60	50
38	121	105	78	81	57		49			29		
36	142	106	103	70	62	47						
34		151	95	95	82							
32			175	146	114	108	96	84				
30						179	143	123				
28								111	77			
26								132	95	90	79	
									104	99	80	
24									127	103	97	64

¹ Each average represents 6 rats.

TABLE 2. AVERAGE¹ LEVELS OF BLOOD GLUCOSE (MG. %) IN THE EVISCERATE RAT AT THE END OF 3 HOURS OF INFUSION OF GLUCOSE WITHOUT INSULIN

TEMPERATURE OF AIR, °C.	GLUCOSE LOAD, MG/100 HR.										
	28	26	24	22	20	18	16	14	12	10	8
38											
26	156	130	130	104	85						
						154	135	137	111	101	88

¹ Each average represents 6 rats.

This provides an explanation for the differences in levels of blood amino acids in eviscerate rats as reported by Frame and Russell (4) and by Ingle, Prestrud and Nezamis (5). Frame and Russell found high values for blood amino acids, and, although they did not indicate the temperature at which their studies were carried out, it was later stated (3) to have been 38°C. Ingle, Prestrud and Nezamis kept eviscerate rats at 26°C. and found comparatively low values for blood amino acids. Chen *et al.* (6) noted a striking effect of temperature upon the sensitivity of the mouse to convulsive doses of insulin.

The problem of temperature control in animal experimentation is not limited to the regulation of heat in the surrounding air as can be illustrated by two experiences of the authors during the past year. In all of our studies of the work performance of

the anesthetized rat, the animals were enclosed in a box with temperature constant at 28°C. During a period of 15 years the procedure of stimulating one gastrocnemius muscle 3 times per second was used. Under these conditions no effect upon work or survival was observed when the hair was clipped from the rat incidental to surgery. During the past 2 years the procedure of stimulating all of the musculature of both legs (7) has been used. Under these conditions the temperature of the unshaved rat was rapidly elevated above normal and it was found necessary to clip the hair from the body in order to accentuate the loss of heat and thereby permit optimal work performance and survival.

As a second example, it was found that normal rats kept in a constant temperature room show a significant increase in nitrogen excretion when the hair is shaved in preparation for surgery. A similar effect was noted on unshaved animals when large mesh screen covers for the metabolism cages were substituted for covers having solid sides with a screen top. The importance of such factors which affect the rate of heat loss of experimental animals can be readily understood when the attention of the investigator has been directed to them, but the present authors became aware of these factors by accident and not by anticipation.

SUMMARY

The tolerance of male eviscerate rats for glucose administered by constant intravenous injection was studied over a temperature range of 24 to 38°C. for a period of 3 hours. The poikilothermic behavior of eviscerate rats was confirmed and under these conditions there was a close correlation between the temperature of the surrounding air and the glucose load which the rat was able to tolerate. In *experiment 1*, rats were given glucose with insulin. At a temperature of 24°C., tolerance was represented by a glucose load of 60 mg. of glucose per 100 grams of rat per hour (60/100/hr.). At a temperature of 38°C., tolerance was represented by a glucose load of 150/100/hr. In *experiment 2*, rats were given glucose without insulin. At 26°C., the glucose load of 10/100/hr. best represented tolerance and at 38°C., the glucose load which best represented tolerance was 22/100/hr.

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EFFECTS OF THE HYPERGLYCEMIC-GLYCOGENOLYTIC FACTOR (HGF) FOUND IN INSULIN PREPARATIONS¹

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THIS study was undertaken to evaluate certain aspects of the action of the hyperglycemic-glycogenolytic factor (HGF) present in various commercial insulin preparations. The pertinent literature prior to 1945 has been summarized in the monographs by Hill and Howitt (1), Jensen (2) and de Duve (3). The intravenous injection (single or repeated) of insulin in various vertebrates causes an initial, slight, temporary elevation of the blood sugar, which is then followed by the typical hypoglycemic response. However, from the time of injection of the insulin, there is a fall in liver glycogen.

Prolonged hyperglycemia is noted in the report of Jourdonais and Bruger (4) who gave a single massive, intravenous dose of insulin powder to rabbits. Bridge (5) showed that the addition of LILLY insulin (1.5–6.0 U/kg/hr.) to an infusion of glucose and saline in rabbits, would cause a more marked, prolonged hyperglycemia than the control glucose infusion. This was extended by de Duve *et al.* (6, 7) who showed that continuous infusion of LILLY insulin (16–23 U/kg/hr.) caused a prolonged hyperglycemia. This effect was not noted when NOVO insulin (manufactured by the Novo Laboratories in Copenhagen) was used. They concluded that this insulin did not contain appreciable amounts of an HGF. Olsen and Klein (8), using single intravenous injections in cats, corroborated this distinction between NOVO and other commercial insulins.

Sutherland and Cori (9) were able to show that the *in vitro* glycogenolytic action of insulin, reported by Shipley and Humel (10), was due to the HGF and not to the insulin itself. NOVO insulin and insulin recrystallized by the method of Abel *et al.* (11) had none or minute quantities of the HGF. Pincus *et al.* (12), however, reported that NOVO insulin does contain the HGF, although in very small amounts. Weisberg, Friedman and Levine (13) demonstrated that the constant infusion of 0.1 U/kg/hr. of NOVO insulin into the splenic vein of dogs caused an initial, temporary but not a prolonged hyperglycemia. The hyperglycemic effect of NOVO insulin was not seen when the infusion was given via a systemic (femoral) vein.

Sutherland and Cori (9), as had Burger and Kramer (14), demonstrated that the HGF was a protein. Sutherland and de Duve (15) have shown the HGF to be present in the pancreas and in the mucosa of the upper part of the stomach and of the duodenum and ileum. They suggest the possibility, which is not definitely proven, that the alpha-cells or the argentophil cells (present in the gastro-intestinal tract and pancreas) may be the source of the HGF.

In the course of corroborating the results of de Duve (6, 7), we became interested in determining the various conditions which are involved in eliciting the hyperglycemic response of the HGF. In addition, we were interested in investigating the relationship of the HGF to the adrenal gland because of the 'insulin-epinephrine antagonism' and the statement of Wichels and Lauber (16) that the hyperglycemic effect of insulin ('insulin-diabetes') is due to the action of epinephrine.

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METHODS

Series I (No anesthesia). Fifty-four adult rabbits (51 females and 3 males) were used. The control blood sugars averaged 96 mgm. per cent (range 58-158). Animals were allowed food and water *ad lib.* ('non-fasted') except those ('fasted') which were allowed access only to water for 24 hours. The animals were given the various solutions by the intravenous infusion technique as used in unanesthetized patients. The experimental period lasted 6 hours. Twenty-six cc. of saline/hour were infused by a constant injection pump via the marginal ear vein. Insulin was given at doses varying between 1 to 50 U/kg/hr. in the saline infusion. Blood samples were analyzed in duplicate by the Somogyi-Shaffer-Hartmann method (17, 18).

Series II (Pentobarbital anesthesia). In 17 adult female rabbits under pentobarbital anesthesia, the pre-operative blood sugar averaged 89 mgm. per cent (range 62-151) and the one hour post-operative value (at start of the infusion) was 126 mgm. per cent (range 79-250). Operations performed were either bilateral adrenalectomy or a sham abdominal operation. Two per cent procaine solution was infiltrated into the region surrounding the adrenals prior to ligation and adrenalectomy. A saline infusion was started one hour after adrenalectomy at a constant rate of 52 cc/hr. for one hour. Other conditions were the same as for *Series I*.

The blood sugar level at the start of the infusion was taken as the control value of 'zero'. The curves are drawn through the average values for all animals used in any experiment and represent the changes expressed in mgm. per cent from the control value.

RESULTS

Our preliminary experiments demonstrated the difference between NOVO and other commercial preparations of insulin, as noted by de Duve *et al.* (6, 7). A more rapid fall in blood sugar resulted when NOVO was used. The drop in blood sugar was greater and remained lower for a longer period of time in contrast to other insulins (fig. 2).

A control experiment, using 6 rabbits in which no infusion was given for the entire 6-hour period, showed the blood sugar value to rise approximately 20 mgm. per cent by the end of 30 minutes. The blood sugar remained relatively constant at this value for the remainder of the experimental period.

Series I. Figure 1 presents the results in 37 normal, non-fasted, non-anesthetized, adult rabbits injected with 1, 5, 10, 15, 25 and 50 U/kg/hr. of LILLY insulin in 26 cc. of saline/hour. It can be seen that the same hypoglycemic levels are reached in 4 hours for the 1, 5 and 10 unit doses. (The rise at the sixth hour for the one unit curve is due to the death of 3 of the 4 rabbits at the end of 5 hours.) The presence of the HGF is noted in the different rates of fall in blood sugar for these three curves. The 15 and 25 unit curves show a prolonged hyperglycemic effect. However, the 50 unit curve shows a slight, initial hyperglycemia at one hour and then hypoglycemia ensues but at a slower rate than for the 1, 5 and 10 unit curves.

Figure 2 demonstrates the difference between LILLY and NOVO insulin when given at a dose level of 15 U/kg/hr. In addition, the influence of the nutritional status is shown in the two curves for LILLY insulin—one, non-fasted rabbits and the

other, fasted for 24 hours. Fasting for 24 hours prevents or abolishes the hyperglycemic effect noted in the non-fasted rabbits given LILLY insulin at 15 U/kg/hr.

At a higher dose (25 U/kg/hr.), the lack of hyperglycemic response is also seen in the fasted group (fig. 3). In addition, figure 3 presents data showing that the administration of insulin to well fed rabbits via the subcutaneous route does not result in hyperglycemia but only in a pronounced hypoglycemia. A response to SQUIBB insulin is included to show the variability in content of the HGF present in commercial insulins. SQUIBB insulin given to a well fed rabbit resulted only in hypoglycemia but not to the same extent as that seen when LILLY insulin was given intravenously to fasted rabbits or to well fed rabbits via the subcutaneous route.

Series II. In our preliminary experiments it was found that manipulation (e.g. withdrawal of blood) of the vena cava near the adrenal glands would result in a progressive rise in blood sugar (118 mgm. per cent increase at the end of one hour). When 2 per cent procaine was infiltrated around the adrenals, the blood sugar remained at about the control level for the entire one hour period, despite the same manipulations.

Figure 4 presents the data on the 17 anesthetized rabbits injected with saline at the rate of 52 cc/hr. for the one hour experimental period. LILLY insulin was given in the saline infusion at a dose of 15 U/kg/hr. During the one hour 'recovery' period after the operation there was a rise in the blood sugar levels. In figure 4 the zero control value is the level of the blood sugar at the end of the recovery period, when the infusion was started. The increases from the pre-operative to the control level were: a) fasted, sham operated, 13 mgm. per cent; b) non-fasted, sham operated, 20 mgm. per cent and c) non-fasted, adrenalectomized, 60 mgm. per cent.

Infusion of saline alone to well fed rabbits with sham operation or adrenalectomy resulted in a drop of 26 and 38 mgm. per cent, respectively, at the end of the hour. LILLY insulin infused into well fed rabbits with sham operation caused a blood sugar rise of 135 mgm. per cent at the end of the hour, whereas in the adrenalectomized only a rise of 38 mgm. per cent occurred. LILLY insulin given to fasted rabbits caused a drop of 22 mgm. per cent in contrast to the increase of 135 mgm. per cent in the well fed rabbits.

DISCUSSION

Certain conclusions can be drawn from our experiments, as to the factors which are concerned in the manifestation of hyperglycemia due to the HGF, found in commercial insulin preparations. These conclusions are listed below and will be discussed in relation to the pertinent findings in the literature.

Intravenous administration, especially via the hepatic portal system, of commercial insulin produces the most marked hyperglycemia. The HGF, which is a protein, is inactivated or broken down when given subcutaneously. Fasting abolishes or greatly reduces the tendency to hyperglycemia. This is related to the level of the liver glycogen. It is presumed that, in addition to an adequate liver glycogen, some factor from the adrenal gland (medulla or cortex) is necessary for the action of the HGF to be evident. Anesthesia enhances the hyperglycemic effect. Commercial

insulin preparations contain an HGF, the content of which varies with different brands and/or lots of manufacture. The degree of hyperglycemia bears no consistent relationship to the dose of HGF administered.

The hyperglycemic effect of commercial insulin has usually been noted with single or repeated intravenous injections. Our data (fig. 1) show the hyperglycemic response when commercial insulin is given by continuous intravenous infusion, thus confirming de Duve *et al.* (6, 7). The effect is greater if the HGF reaches the liver first (via the hepatic portal circulation) rather than after passing through the general circulation (19, 20). We have observed the effect of infusing LILLY or NOVO insulin into the splenic and femoral veins of dogs (13). The initial hyperglycemic effect is less and of shorter duration with NOVO than with LILLY insulin when given via the splenic vein. Via femoral vein injection, no initial hyperglycemia is seen with NOVO, and LILLY gives a hyperglycemic response which is not as marked

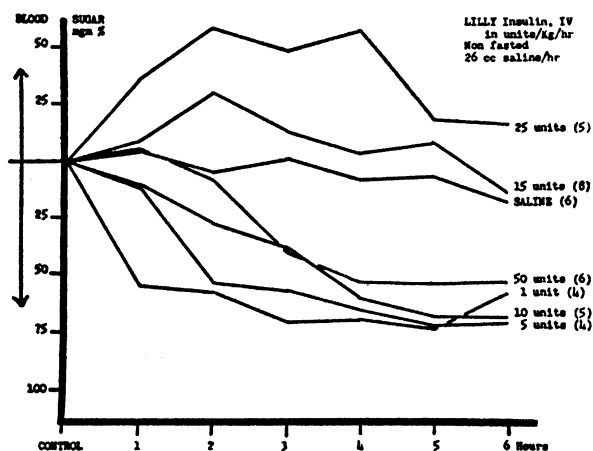


Fig. 1. EFFECT OF VARYING DOSE LEVELS of intravenous Lilly insulin/kg/hr. on blood sugar of adult, well fed, non-anesthetized rabbits. Insulin was administered by a constant injection pump in 26/cc. saline/hr. Number of animals in each experiment given in parentheses.

as via splenic (13). Therefore, in order to have its maximum effect, the HGF must reach the liver directly via the hepatic portal system.

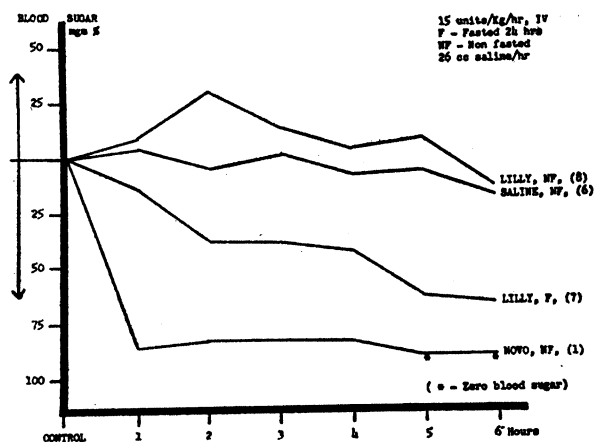
Subcutaneous continuous infusion of LILLY insulin did not result in hyperglycemia (fig. 3). Olsen and Klein (8) did not find the typical initial hyperglycemia after the single subcutaneous or intramuscular injection of insulin. Pincus *et al.* (12) used both unmodified and inactivated (method of Sutherland and Cori (9), which leaves the HGF unaltered) insulin and found that subcutaneous and intraperitoneal injection did not produce any significant hyperglycemia. It was noted by Sutherland and de Duve (15) that the isolated HGF was destroyed at an appreciable rate when it was added to blood. It seems likely, therefore, that the HGF which is a protein (9, 14) can be inactivated very quickly in the body. This agrees with the preceding discussion, that the maximum hyperglycemic effect is seen when the HGF reaches the liver directly via the shortest route.

It is well known from perfusion (21), *in vitro* (9, 10) and *in vivo* (5, 22-24) experiments that commercial insulin causes a breakdown of liver glycogen. In our experiments, the hyperglycemia was noted only in well fed rabbits and not in rabbits

fasted for 24 hours (figs. 2 and 3). This agrees with Wichels and Lauber (16) who noted a more marked hyperglycemia in well fed rabbits. This is probably correlated to the level of the liver glycogen, which is low after fasting. Pincus *et al.* (12) reported that the presence of the liver is necessary for the evocation of the hyperglycemic response. With fasting there is a decreased liver insulinase activity and less inactivation of insulin (25). The relatively larger concentration of insulin (due to less insulinase activity) may mask the effect of HGF, especially with a lower liver glycogen, due to fasting.

From the evidence presented in fig. 4 it seems that the presence of the adrenal gland is necessary for the hyperglycemic effect to be marked. It may be argued that the liver glycogen in acute adrenalectomy is too low for the HGF to have its effect. However, various reports (5, 22, 23) state that the adrenalectomized animal responds to insulin as does the normal, with a reduction in liver glycogen. Reid (23) noted

Fig. 2. COMPARISON OF LILLY AND NOVO INSULIN at dose of 15 U/kg/hr. Comparison of feeding (NF) vs. fasting (F). Other conditions as for fig. 1.



that the blood sugar did not rise in the adrenalectomized cat, even though the liver glycogen had fallen. Basiliou and Zell (26) reported that, after the removal of one or both adrenals, the hyperglycemic effect of insulin was not noted.

The liver glycogen in 2 rabbits was determined, one hour after bilateral adrenalectomy, and was 0.65 and 0.16 per cent. We found (27) that normal rat liver slices were able to produce a 40 per cent increased output of glucose in the presence of the HGF (inactivated insulin, (9)), whether the liver glycogen was 2.55 or 0.29 per cent. However, liver slices taken from a rat 36 hours after adrenalectomy, with the liver glycogen at 0.75 per cent, did not have any increased output of glucose in the presence of the HGF (27).

It seems, therefore, that in addition to a reserve supply of liver glycogen, some factor from the adrenal medulla or cortex is necessary for the action of the HGF to be evident.

The data presented in figure 4 show that anesthesia causes a more marked hyperglycemic response to the continuous infusion of insulin (15 U/kg/hr.). With pentobarbital anesthesia and sham operation, the infusion of insulin caused an average

rise in blood sugar of 135 mgm. per cent at the end of one hour (fig. 4), in contrast to the non-anesthetized, non-operated animals which had an average increase of 9 mgm. per cent at the end of one hour's infusion (fig. 2). Olsen and Klein (8) had noted that the single injection of insulin produced a more marked hyperglycemic response in the anesthetized cat. An operative procedure, especially adrenalectomy, caused a rise in blood sugar even before insulin was administered to the rabbits. Infiltration of the adrenal area with procaine abolishes the (reflex) hyperglycemia due to manipulation (e.g. withdrawal of blood from the vena cava).

From our data and those in the literature, it is apparent that the HGF is present in varying degree in different brands and/or lots of manufactured insulin. NOVO insulin, reported to be free of the HGF, has been shown to contain some HGF (12, 13).

Our data show that there is no consistent relationship of the hyperglycemic effect to the dose of insulin administered. In relatively small doses (less than 10

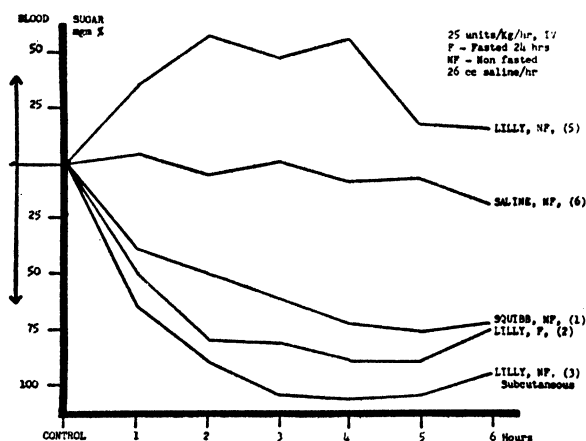


Fig. 3. COMPARISON, AT 25U/KG/HR., of different brands of insulin, nutritional status and route of administration. Other conditions as for fig. 1.

U/kg/hr.) the presence of the HGF is demonstrated even though there is a pronounced hypoglycemia (fig. 1). With higher doses (15 and 25 U/kg/hr.) the hyperglycemic effect is progressive and with 50 U/kg/hr., hypoglycemia is noted (fig. 1). However, if 50 U/kg/hr. of inactivated (9) insulin are given, persistent hyperglycemia does result (27). This agrees with Pincus *et al.* (12) and Sutherland and Cori (9) who found that inactivated insulin produced a more marked and more sustained rise in blood sugar than did unmodified insulin.

The possibility of the HGF being a second pancreatic hormone has long been entertained but not proven (see 1 and 2 for early literature). The work of Sutherland and de Duve (15) is very suggestive that the HGF comes from the alpha-cell of the pancreas or the argentophil cell of the pancreas and gastrointestinal tract. Heard *et al.* (28) did not prove the existence of an alpha-cell hormone; they were able to extract a fraction from the pancreas which caused liver glycogenolysis and hyperglycemia. Zimmermann and Donovan (29) were unable to neutralize the hypoglycemic action of insulin by the *subcutaneous* injection of the HGF. However, Pincus (30) states

that the *intravenous* administration of the HGF can prevent the hypoglycemia produced by small doses of insulin. This agrees with our previous discussion of the inactivation of the HGF by the body tissues.

In addition there are other data, animal and clinical, which tend to support the idea that a factor of this nature may act to antagonize the hypoglycemic effect of insulin. Young (31) has shown that the permanent diabetes of dogs, due to the administration of anterior pituitary extracts, required more insulin per day for control than did dogs made diabetic by pancreatectomy. Dragstedt *et al.* (32) showed that partially depancreatized dogs required more insulin than did completely depancreatized dogs. Thorogood and Zimmermann (33) reported that alloxanized dogs required more insulin than did those with complete pancreatectomy. Only the beta-cells of

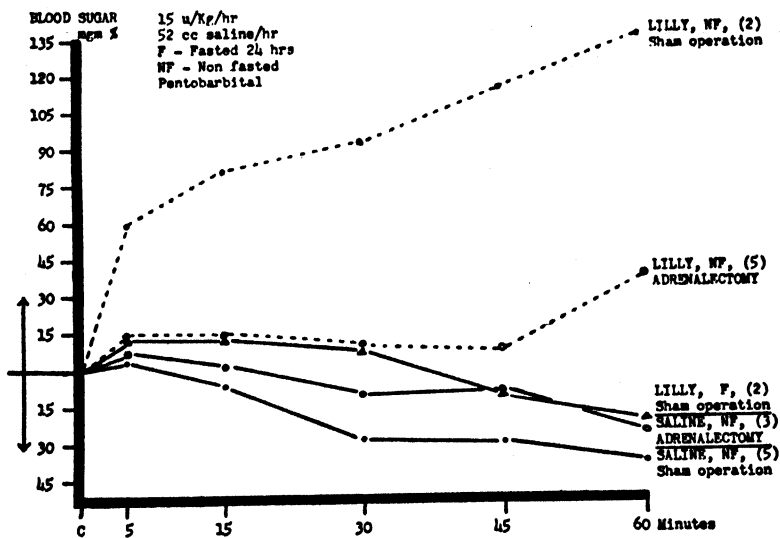


Fig. 4. LILLY INSULIN administered at 15U/kg/hr. in 52 cc. saline for 1 hr. by constant injection pump to anesthetized adult rabbits. Comparison of bilateral adrenalectomy vs. sham abdominal operation.

the pancreas are destroyed by the administration of alloxan or anterior pituitary extracts. The clinical data, as well, tend to show that total pancreatectomy in man will result in a diabetic state which is less severe and requires less insulin than the moderately severe diabetes mellitus (34, 35). Although it is hazardous to judge accurately the severity of diabetes by insulin requirement, the above quoted work is sufficiently consistent to indicate that a factor antagonistic to the hypoglycemic action of insulin may be present in the pancreas.

However, before we can call the HGF a hormone secreted by the alpha-cell (or the argentophil cell of the pancreas and gastro-intestinal tract) we need clear cut evidence that it is secreted into the blood. Sutherland and de Duve (15) were unable to demonstrate the presence of the HGF in the blood of the pancreaticoduodenal vein from the normal or perfused pancreas. We were unable to demonstrate the HGF

in pancreatico-duodenal vein blood of dogs, during the first hyperglycemic phase of alloxan action (36).

SUMMARY

Our own data and a review of previous work lead us to make the following conclusions: Commercial insulin preparations contain a hyperglycemic-glycogenolytic factor (HGF), the content of which varies with different brands and/or lots of manufacture. The hyperglycemic effect is most marked when the factor reaches the liver via the shortest route. The HGF causes glycogenolysis of the liver glycogen to glucose. The HGF, which is a protein, is inactivated or broken down when given subcutaneously. The degree of hyperglycemia bears no consistent relationship to the dose of HGF administered. Anesthesia enhances the hyperglycemic effect. Fasting abolishes or greatly reduces the tendency to hyperglycemia; this is related to the level of liver glycogen. In addition to adequate liver glycogen, it is presumed that some factor from the adrenal gland (medulla or cortex) is necessary for the hyperglycemic effect of the HGF. The physiological significance of the HGF is discussed and leads to the conclusion that, as of the present, there has been no clear cut demonstration to prove that the HGF is a hormone and/or that it is secreted by the alpha-cell or the argen-tophil cell.

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NATURE OF THE ACTION OF INSULIN ON THE LEVEL OF SERUM INORGANIC PHOSPHATE¹

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IT IS well known that the administration of dextrose, insulin or epinephrine to normal animals produces a diminution in the level of the serum and blood inorganic phosphate (1-4). Soskin, Levine and Hechter (5) showed that in the absence of the pancreas, neither glucose nor epinephrine would produce a fall in the inorganic phosphate of the blood. It was therefore concluded that the fall of blood inorganic phosphate is a specific and direct action of insulin. In the normal animal glucose and epinephrine exerted this effect by causing extra insulin secretion.

Recent work in this laboratory (6) has shown that the untreated depancreatized dog has a 'normal' tolerance for intravenous fructose. Fructose disposal or tissue entry, therefore, seems not to be affected by the presence or absence of insulin. Wierzechowski (7) demonstrated a significant fall in blood inorganic phosphate following the intravenous administration of fructose to *normal* dogs.

This paper reports experiments on the effect of the intravenous administration of fructose to totally depancreatized dogs, on their blood inorganic phosphate levels. A rapid and significant fall in phosphate under these conditions would indicate that this effect is due to the rate of entry of a hexose into tissues and not necessarily to a participation of insulin. Since a rise in the level of glucose results in greater utilization even after pancreatectomy (8), we have also tested the effect of larger amounts of glucose than previously employed, on the phosphate level, in the complete absence of insulin.

METHODS

Ten depancreatized dogs were used, ranging in weight from 6 to 19 kg. The animals were fasted and maintained without insulin for 48 to 96 hours before the experiments were conducted. One third of a gram of fructose per kilogram body weight in 15 to 20 cc. of normal saline was injected intravenously. Blood glucose, fructose and inorganic phosphate were measured at 0, 5, 15, 30, 60, 90, 120 and 180 minutes after injection of the hexose. Blood glucose was measured by the Somogyi-Shaffer Hartmann method (9), fructose by the method of Corcoran and Page (10) and inorganic phosphate according to Fiske and Subbarow (11).

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RESULTS

Figure 1 demonstrates typical changes in blood glucose, fructose and inorganic phosphate which occurred following the administration of the test dose of fructose. It is readily seen that the fructose is rapidly disposed of, so that by 60 minutes post-injection, only 5 mg/100 cc. of blood remains. The blood glucose level rises moderately for 60 to 90 minutes, then returns to its original level in about 3 hours. In every case, there was a decline in the inorganic phosphate of between 0.5 and 2.0 mg/100 cc., averaging 1.1 mg/100 cc. (fig. 2). In 2 non-diabetic dogs, the declines in phosphate were 1.1 and 0.6 mg/100 cc., respectively. Numerous previous studies in this laboratory have shown that, in dogs, any variation in phosphate of greater than 0.4 mg/100 cc. is significant.

In the study reported by Soskin, Levine and Hechter (5), the maximum decrease in blood inorganic phosphate produced by glucose in 7 depancreatized dogs was

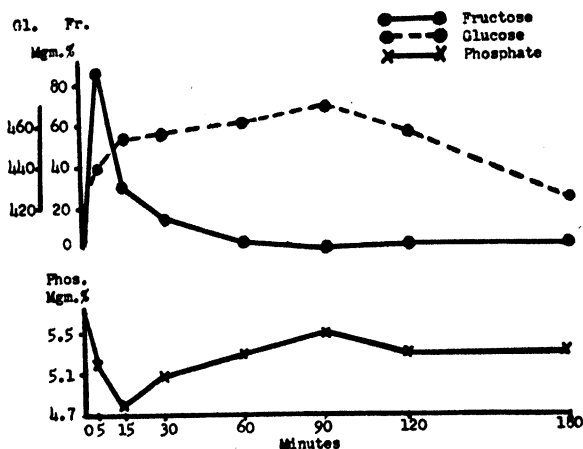


Fig. 1. EFFECT OF A SINGLE I.V. INJECTION OF FRUCTOSE ($\frac{1}{3}$ gm/kg.) on the level of blood inorganic phosphate and glucose of the untreated depancreatized dog.

0.4 mg/100 cc. When glucose was administered to normal dogs (1.75 gm/kg. body weight), decreases in phosphate of 0.5 to 1.2 mg/100 cc. occurred, with an average of 0.8 mg. It is apparent that even with a smaller dose ($\frac{1}{3}$ gm/kg. body weight), fructose is as effective in producing a drop in phosphate in the diabetic animal, or slightly more so, than is glucose in the normal animal (table 1).

However when larger amounts of glucose are given a phosphate drop occurs in the absence of insulin. In 4 experiments on 3 depancreatized dogs, glucose was injected by constant infusion (2.0–3.0 gm/kg/hr. for two hours) producing maximum blood glucose levels of 568 to 957 mg/100 cc. In each instance there was a decline of 0.7 to 1.7 mg/100 cc. in the blood phosphate level (table 1). One dog was infused, 44 hours after pancreatectomy, with 3.4 gm/kg/hr. for 90 minutes, reaching a blood glucose level of 853, no drop in phosphate occurring. The same dog, however, 96 hours later demonstrated a fall of 0.9 mg. in phosphate when 3 gm/kg/hr. for 2 hours were administered.

DISCUSSION

From the data presented above, it is apparent that even in the complete absence of insulin, i.e. in the untreated depancreatized animal, fructose injection

produces a significant drop in the level of blood inorganic phosphate. Fructose has been demonstrated to be disposed of, i.e. to enter the tissues, at a rapid rate, in the absence of insulin, this rate not being further increased by the administration of insulin (6). In the normal animal, or in the depancreatized animal supplied with insulin, glucose also enters the tissues at a normal rate, and this is accompanied by a phosphate fall. When, however, insulin is withdrawn, glucose uptake is impaired and under these circumstances no decline in phosphate is seen. It can be readily reasoned, therefore, that the fall in blood phosphate is a function of the rate of

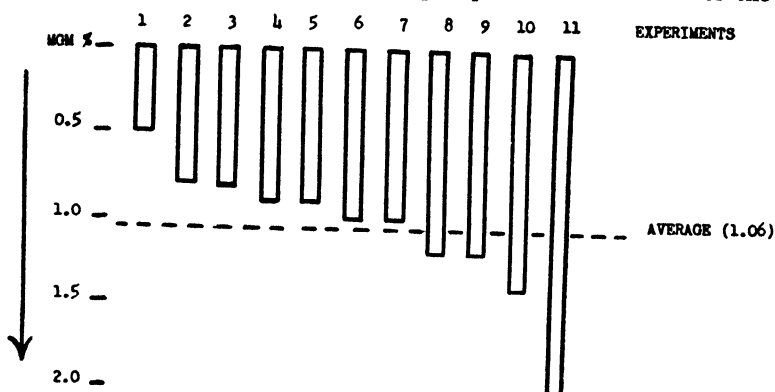


Fig. 2. DECREASE OF BLOOD INORGANIC PHOSPHATE following fructose administration to untreated depancreatized dogs. Each column represents the decrease in inorganic phosphate in mg. % from the initial control values.

TABLE I

NO. AND CONDITION OF DOGS USED	AMOUNT AND TYPE OF HEXOSE INJECTED	DECREASE IN SERUM INORG. PHOSPHATE			REFERENCE
		Minim.	Maxim.	Average \pm S.E.	
5, Normal 7, Depancreatized	Glucose (1.75 gm/kg.)	0.5	1.2	0.8 \pm 0.04	<i>Am. J. Physiol.</i> 134: 40, 1941
	Glucose (1.75 gm/kg.)	0.0	0.4	0.2 \pm 0.02	
10, Depancreatized 4, Depancreatized	Fructose (0.33 gm/kg.)	0.5	2.0	1.1 \pm 0.12	Present data
	Glucose (2-3 gm/kg/hr., for 2 hours)	0.7	1.7	1.0 \pm 0.20	

entry of the hexose into the tissues. This decline is independent of the action of insulin, except, as in the case of glucose, when insulin speeds the rate of uptake of the hexose.

It has been shown that at higher levels of blood sugar, dextrose utilization will proceed in normal fashion in the depancreatized dog (8). It would appear likely that, if the phosphate fall is produced by the entry of hexose into tissues, it ought to occur when glucose is 'pushed' into the cells of a diabetic animal, by administering it at a very high blood level. This is the case as seen by the data in table I, which compares the results using different amounts of glucose with those following fructose infusion.

We must therefore change our previous interpretation that the fall of phosphate

is due to a direct action of insulin, in favor of the concept that it is an accompaniment of the entry of the blood hexose into cells. Insulin can be related only indirectly, namely, when it accelerates this entry into cells, as it does with glucose.

CONCLUSIONS

The intravenous administration of fructose to untreated depancreatized dogs produces invariably a fall in blood inorganic phosphate. The rate of entry of fructose from the blood into the tissues of such animals does not differ from that in normal dogs. The rate of disappearance of injected glucose from the blood is slower in the absence of insulin. Moderate amounts of glucose do not lead to a significant fall in blood inorganic phosphate. At very high blood glucose levels the rate of tissue entry of this hexose is increased and then leads to a significant fall in blood inorganic phosphate. The fall in blood inorganic phosphate is a consequence of rapid entry of hexoses into tissues and is not a primary, direct action of insulin.

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INSULIN SENSITIVITY OF THE EXTRAHEPATIC TISSUES OF THE ADRENALECTOMIZED RAT¹

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THE hypophysectomized or adrenalectomized animal exhibits a greatly increased sensitivity to the hypoglycemic effect of insulin, as compared to normal intact animals (1, 2). This has been confirmed for several species including the mouse, rat, rabbit, guinea pig, dog, cat, monkey, toad and man. However, the mechanism of the increased sensitivity of these hypophysectomized or adrenalectomized animals to insulin is not completely understood. On theoretical grounds any or all of the following factors may be responsible for this phenomenon: a) inadequate counter-regulatory responses (i.e. glycogenolysis and gluconeogenesis) to hypoglycemia by the liver of the hypophysectomized or adrenalectomized animal; b) a decreased rate of inactivation of insulin by the blood and tissues of the hypophysectomized or adrenalectomized animal; c) the absence in the hypophysectomized or adrenalectomized animal of an anti-insulin factor which antagonizes the action of insulin in the extra-hepatic tissues of the normal animal. In this connection, it is well known today that the C-11 oxysteroid hormones of the adrenal cortex are directly antagonistic to the blood sugar lowering action of insulin; i.e. they increase the resistance to a given dose of insulin. The site of action of this anti-insulin effect is unknown.

In order to determine which of the above mechanisms is primarily responsible for the development of the increased sensitivity to insulin in the hypophysectomized or adrenalectomized animal, it is necessary to know whether or not the liverless or eviscerated adrenalectomized or hypophysectomized animal also manifests an increased sensitivity to insulin. In other words, it is important to know whether the site of action for the mechanism of this phenomenon resides in the liver, the peripheral tissues or in both. Bennett and Roberts (3) have performed such experiments in hypophysectomized eviscerated rats. They found that hypophysectomized eviscerated rats are 2 to 4 times more insulin-sensitive than normal eviscerated rats. These results may be compared to the results of other experiments in which it was shown that *intact* hypophysectomized rats were 12 to 16 times more sensitive to the hypoglycemic action of insulin than normal rats (4).

Since no information of this nature is available for adrenalectomized animals, a series of experiments was conducted to determine whether or not eviscerated adrenalectomized rats were more sensitive to the hypoglycemic action of administered insulin than normal eviscerated rats.

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PROCEDURE

The animals used in these experiments were female rats of the Long-Evans strain weighing between 180 and 250 grams. The diet consisted of Purina Fox Chow pellets, *ad libitum*.

Bilateral adrenalectomy was performed by the lumbar approach under ether anesthesia. Evisceration was done 5 to 10 days after adrenalectomy. In order to determine the optimal substitutional therapy (in respect to NaCl and water), which would maintain the adrenalectomized rats in the best possible condition and thus minimize the shock following evisceration, some animals were maintained with 1½ per cent salt solution substituted for the drinking water, and some with daily intramuscular injections of 1 to 3 mg. of desoxycorticosterone acetate in oil (DCA)^{*} during the period between adrenalectomy and evisceration. The animals receiving DCA were not given salt in their drinking water.

Evisceration was performed under ether anesthesia by the functional evisceration technique. In addition, a partial hepatectomy was done by tying off and removing the major portion of the 3 largest lobes of the liver. All preparations in which there was more than a trace of bleeding were discarded, inasmuch as it has been shown by others (5, 6), and confirmed by ourselves, that hemorrhage accelerates the fall in blood sugar in the eviscerated rat despite fluid replacement with normal saline.

Samples for blood sugar determinations were obtained by collecting 0.1 cc. of blood from the cut tip of the tail at the desired intervals. Serial blood sugar determinations (done by the method of Nelson, 7) were performed so that curves of the rate of fall of the blood sugar following evisceration could be constructed. Blood sugars were obtained at 0, 20 and 60 minutes after the completion of the evisceration, and hourly thereafter until the death of the animal. Terminal blood samples were obtained from the inferior vena cava. The survival times of all of these animals were noted and recorded. All of the animals died in hypoglycemic convulsions.

Insulin was administered intravenously into the inferior vena cava immediately after the completion of the evisceration, and was so diluted with normal saline that a constant volume of 0.5 cc. was given to all animals. All control animals received 0.5 cc. normal saline intravenously. Insulin was given to normal eviscerated animals and to those adrenalectomized animals which had been maintained on 3 mg. DCA daily, and resultant blood sugar curves were compared with the curves obtained in the corresponding control groups of rats which did not receive insulin. The adrenalectomized rats were also given 2 cc. of normal saline subcutaneously each hour in an effort to prevent shock. The normal rats did not receive these additional saline injections.

Finally, a few auxiliary experiments were performed to determine the effect of nephrectomy on the rate of fall of the blood sugar following evisceration of normal and adrenalectomized rats. The adrenalectomized rats used in these experiments were maintained with 1½ per cent salt solution. A functional nephrectomy was performed by ligating both renal pedicles. Curves of the blood sugar fall in the nephrec-

* Desoxycorticosterone acetate (DOCA, Organon, Inc.) in peanut oil was generously supplied through Dr. Kenneth W. Thompson of Organon, Inc., Orange, N. J.

tomized animals were compared with curves obtained in the corresponding control groups.

RESULTS

The survival time of 9 normal eviscerated rats varied from $5\frac{1}{4}$ to $13\frac{1}{2}$ hours with an average of 8.3 hours. All of the animals died in hypoglycemic convulsions. A curve showing the rate of fall of the blood sugar following evisceration was constructed for each of the animals. In general, the steepness of the curve was related inversely to the survival time of the animal.

The survival time of 9 adrenalectomized eviscerated rats which had been maintained on $1\frac{1}{2}$ salt solution varied from 60 to 175 minutes with an average of 108

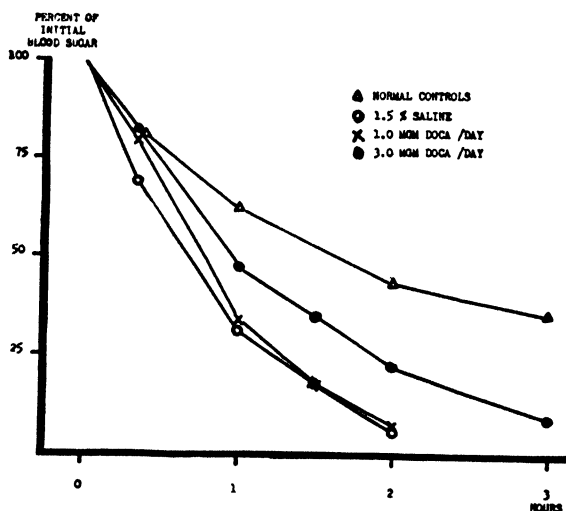


Fig. 1

minutes (1.8 hours). The curve of rate of fall of blood sugar was correspondingly steeper in these animals than in the normal group.

Adrenalectomized rats maintained with 1 mg. DCA per day did not differ appreciably from the salt-treated group in either the survival time or the slope of the curve of blood sugar fall following evisceration (fig. 1). The survival times of this group of animals varied from 90 to 165 minutes with an average of 124 minutes. However, when adrenalectomized animals were maintained on 3 mg. of DCA daily, there was a significant prolongation in the survival time of this group, and correspondingly there was a less rapid fall in the blood sugar following evisceration (fig. 1). The average survival time of this group was 210 minutes (3.5 hours), approximately double that of the preceding 2 groups of adrenalectomized rats, with survival times ranging from 140 to 330 minutes. It should be noted, however, that maintenance of the adrenalectomized animals with this dose of DCA did not completely restore them to the values obtained in normal eviscerated rats. These observations are similar to those of Harrison and Harrison (8), Wells and Kendall (9) and Russell (10), who found that the administration of similar doses of DCA to adrenalectomized or hypophysectomized

animals partially prevented the changes in carbohydrate metabolism resulting from ablation of either the adrenals or the hypophysis. We feel that these observations can best be explained by the salt-retaining and vascular effects of DCA, similar to the beneficial effects on carbohydrate stores demonstrated in adrenalectomized animals maintained by salt alone (11).

Effect of Insulin on the Rate of Fall of Blood Sugar in Normal and Adrenalectomized Eviscerated Rats. The object of these experiments was to determine the minimal dose of insulin injected intravenously which would accelerate the fall of blood sugar in the eviscerated normal and adrenalectomized rats. Adrenalectomized rats maintained with 3 mg. DCA daily were used for these experiments because of the longer survival times and better condition of these animals.

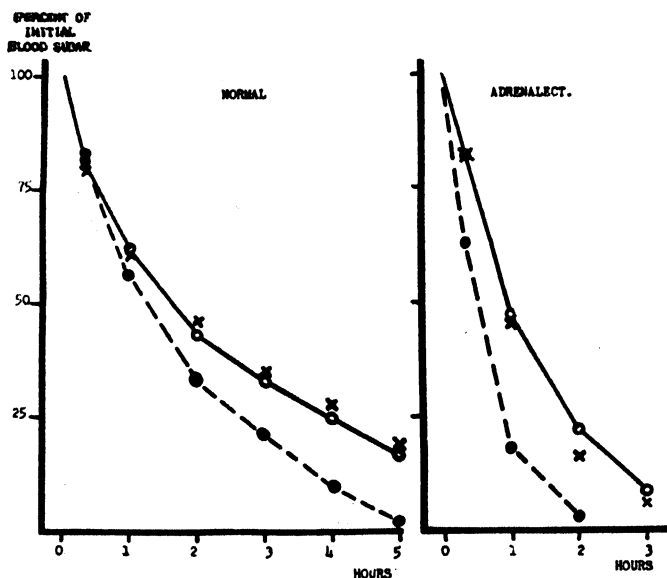


Fig. 2. O = no insulin; X = 0.01 unit/kg.; ● = 0.02 unit/kg.

It was found that the dose of insulin which just exerted a perceptible effect on the rate of fall of blood sugar in some of the animals following evisceration was *exactly* the same in the normal and adrenalectomized groups of animals; this dose was 0.01 u/kg. of body weight (fig. 2, table 1). In the 5 *normal* eviscerated rats given this dose of insulin, there was some degree of acceleration of the fall of blood sugar in 2 of the rats, whereas in the other 3 animals the blood sugar curves were unaffected. This dose of insulin resulted in an accelerated fall of blood sugar in 2 of the 6 adrenalectomized rats tested, the curves of the other 4 animals falling within the limits of the control curves of the adrenalectomized rats *not* given insulin. The average curves for each group of animals (fig. 2) show the negligible effect of 0.01 unit of insulin per kilo on the rate of fall of blood sugar in both the normal and adrenalectomized groups.

However, if the dose of insulin given to these animals is increased to 0.02 u/kg., there is an unmistakable and distinct acceleration of blood sugar fall in both the nor-

mal and adrenalectomized groups. Thus, the dose of insulin which exerts the minimum significant effect on the rate of fall of blood sugar lies between 0.01 and 0.02 U/kg. in both the normal and the adrenalectomized eviscerated rat; 0.02 U of insulin/kg. had a somewhat greater effect in the adrenalectomized group. The sensitivity to insulin of the peripheral tissues of the adrenalectomized rat is about 1.5 times that of the normal control.

Effect of Functional Nephrectomy on Survival Time of Normal and Adrenalectomized Eviscerated Rats. In confirmation of the work of Reinecke (5), it was found that the survival time of normal eviscerated rats was greatly shortened by functional nephrectomy. The survival time of 5 nephrectomized normal eviscerated rats varied from 80 to 165 minutes with an average of 127 minutes (2.1 hours); this represents an average survival time approximately one fourth of that of the normal controls. The rate of fall of blood sugar was correspondingly increased.

TABLE 1. EFFECT OF INSULIN ON BLOOD GLUCOSE OF EVISCERATED, NORMAL AND ADRENALECTOMIZED RATS

NO. OF ANIMALS	IN- SU- LIN U/KG.	BLOOD GLUCOSE (mg. %), MEAN \pm S.D.							MEAN SUR- VIVAL TIME, HR.
		0	20'	60'	120'	180'	240'	300'	
Normal									
9	0.00	101 \pm 9.6	82 \pm 8.0	63 \pm 9.8	44 \pm 13.3	33 \pm 11.7	25 \pm 11.0	17 \pm 8.8	8.3
5	0.01	112 \pm 11.2	89 \pm 8.0	69 \pm 11.4	52 \pm 13.4	39 \pm 14.6	32 \pm 17.0	21 \pm 13.8	
6	0.02	109 \pm 5.7	89 \pm 7.7	61 \pm 10.5	36 \pm 11.0	23 \pm 11.8	11 \pm 11.2	1 \pm 2.2	4.6
Adrenalectomized ¹									
6	0.00	93 \pm 5.0	76 \pm 6.0	44 \pm 5.8	20 \pm 5.0	9 \pm 4.7			3.5
6	0.01	102 \pm 11.1	83 \pm 5.3	46 \pm 10.3	16 \pm 10.0	6 \pm 6.3			2.8
5	0.02	102 \pm 17.2	65 \pm 14.4	20 \pm 15.8	3 \pm 2.8				1.4

¹ The adrenalectomized animals were all maintained with 3.0 mg. DCA/day prior to the experiment.

Functional nephrectomy also resulted in a shortening of the survival time of adrenalectomized eviscerated rats. The survival time of 5 nephrectomized adrenalectomized eviscerated rats ranged from 47 to 80 minutes, with an average of 64 minutes; this represents an average survival time approximately three fifths that of the control adrenalectomized animals. These results indicate that functional nephrectomy increases the rate of fall of blood sugar in eviscerated adrenalectomized rats, but not to the same degree as it does in the normal group.

DISCUSSION

In the absence of added insulin the rate of fall of the blood sugar is greater in the adrenalectomized than in the normal eviscerated rats. It is suggested that this may be due in part to a greater glucose supply by the kidney of the normal than of the adrenalectomized rat. Functional nephrectomy reduces survival time and speeds up the rate of glucose disappearance to a greater degree in the normal rat than in the animals without adrenals.

The experiments reported in this paper indicate that there is only a small difference in insulin sensitivity between *eviscerated* adrenalectomized and normal rats. On the other hand, it has been shown that hypoglycemic convulsions may be induced in *intact* adrenalectomized rats (12), rabbits (13) and cats (14) by doses of insulin which are one tenth to one twentieth of the amount required in intact normal animals of the same species. Grattan, Jensen and Ingle (15) demonstrated that adrenalectomized mice, maintained in good condition by daily subcutaneous injections of 0.1 mg. of desoxycorticosterone acetate and the addition of 0.9 per cent saline to the drinking water, were sensitive to the hypoglycemic convulsive action of insulin at dose levels one fifth of that which produced the same effect in the normal control mice. These observations mean that intact adrenalectomized animals are 5 to 20 times more insulin-sensitive than normal intact animals. These are minimum figures, since they were derived from experiments in which the sensitivity to insulin was gauged by the dose required to produce hypoglycemic convulsions and death in the test animals, and not by the minimum dose of insulin required to show the first perceptible fall of the blood sugar. Our data demonstrating the small degree of insulin hypersensitivity in the *eviscerated* adrenalectomized animal must mean that the insulin hypersensitivity of the *intact* adrenalectomized animal depends largely upon the presence of a functioning liver.

The same conclusion holds true for the hypophysectomized animal with minor reservations. It has been shown by Russell (4) that intact hypophysectomized rats are 12 to 16 times more sensitive to the hypoglycemic convulsive action of insulin than normal control rats. On the other hand, Bennett and Roberts (3) showed that *eviscerated* hypophysectomized rats are only 2 to 4 times more insulin sensitive than *eviscerated* normal control rats. Thus, the major factor determining the difference in the degree of insulin sensitivity between the *eviscerated* and intact hypophysectomized animal must again reside in the presence of a functioning liver. That there is a direct action on the peripheral tissues by the hypophysis and/or adrenal may also be concluded from these experiments, but this effect is quantitatively of less importance than is the hepatic factor. The possibility of a peripheral factor in the insulin hypersensitivity of hypophysectomized animals is also suggested by the work of Himsworth and Scott (16) who demonstrated that an anterior pituitary extract would inhibit the hypoglycemic effect of insulin in hepatectomized rabbits, although it did not alter the rate of the spontaneously occurring hypoglycemia. However, we wish to emphasize that this peripheral effect is secondary in importance to the hepatic factor.

Grattan, Jensen and Ingle (15, 17) have shown that the anti-insulin activities of the adrenal cortex and of the anterior pituitary are both due to the C-11 oxygenated steroids of the adrenal cortex. These authors have furthermore shown that the anti-insulin effect of these substances is associated with an increase in liver glycogen. A variety of clinical and laboratory investigations has pointed to the fact that the C-11 oxysteroids increase hepatic gluconeogenesis and that this effect is dependent upon the action of these steroids in mobilizing endogenous protein (18) and fat (19) for catabolic breakdown. Strong supportive evidence for this view is

provided by the observations that catabolism of exogenous protein and fat proceeds normally in force-fed adrenalectomized and hypophysectomized animals (18, 20).

Hence, it is most probable that the anti-insulin activity of the adrenal cortex is exerted primarily through its ability to increase the rate of conversion of non-carbohydrate foodstuffs to glucose.

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RELATIONSHIP OF THE ADRENAL CORTEX TO INHIBITION OF GROWTH OF HAIR BY ESTROGEN¹

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BOTH estrogens and adrenocortical compounds possess the capacity to suppress the growth of hair. Systemic treatment with estrogens has this effect in rats (1-5) and dogs (6, 7). Other conditions involving high levels of circulating estrogen in dogs are accompanied also by varying degrees of alopecia (8-10). Likewise, growth of hair is inhibited by feeding adrenocortical compounds to normal rats (11), by injecting adrenocortical substances into adrenalectomized rats (12) or by injection of hypophyseal adrenocorticotropin into normal rats (13). Furthermore, the same end may be achieved locally by placing adrenocortical extracts, 11-dehydro-17-hydroxycorticosterone (14, 15) or 11-desoxycorticosterone (16) directly on the skin. Conversely, acceleration in the rate of growth of hair insues after adrenalectomy (17-19). It is, then, pertinent to discover whether intact adrenal glands are essential to the growth-inhibiting action of estrogen, and the following experiment demonstrates that a dose of estrogen adequate to suppress growth of hair does not maintain this inhibition in the absence of the adrenal glands.

PROCEDURE

The growth of hair was studied on one side of the body in an area bounded by the mid-dorsal and mid-ventral lines and the anterior and posterior extremities. The pattern of hair growth was sketched at weekly intervals, at which time the hair was clipped from the area under observation.

Adult female rats of the Long-Evans and Wistar strains were divided into the following groups: 1) ovariectomized, oil-treated; 2) ovariectomized, estrogen-treated; 3) adrenalectomized, ovariectomized, oil-treated, and 4) adrenalectomized, ovariectomized, estrogen-treated. Table 1 indicates the number of rats used in each group. In most instances littermates were divided between the 4 groups. Before the adrenals and/or ovaries were excised, *groups 2 and 4* were injected daily with 20 to 40 μ g. of alpha-estradiol dipropionate² and *groups 1 and 3*, with comparable volumes of the solvent, peanut oil. This treatment was continued until all rats in *groups 2 and 4* exhibited a pronounced suppression of hair growth for 3 successive weeks. At this time the operations were performed and daily injections of the hormone and oil solvent continued. The rats were killed 2 to 3 weeks later, at which time a strip of skin extending from the mid-ventral to mid-dorsal line was prepared for histological study. Purina laboratory chow supplemented twice weekly with green vegetables was fed *ad libitum* during the experiment. The adrenalectomized animals were maintained on 1.0 per cent NaCl drinking solution.

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RESULTS

Weight Changes. It is evident from table 1 that although oil-treatment of ovariectomized rats permitted an increase in weight during the course of the experiment, treatment with estrogen held the body weight at a plateau (*group 2*). Adrenalectomy and ovariectomy had an extremely variable effect on body weight, as is indicated by the standard error of 5.8 in the mean percentage change in body weight (*group 3*). It is to be pointed out that this great variation developed in the 3-week period following adrenalectomy. In contrast, suppression of a gain in body weight in *group 2* was maintained by estrogen throughout the entire course of the experiment. Estrogen caused a mean loss of 5.8 per cent in body weight after adrenalectomy. Although estrogens are considered generally to be highly toxic to adrenalectomized rats, at this dosage level a number of the animals tolerated the hormone rather well.

When the weights of the adrenal glands are considered in relation to body weight, it is apparent that estrogen caused an increase in weight, the ratios being .273 and .333 for *groups 1* and *2*, respectively (table 1). The rather significant standard errors of these means are accounted for by the fact that two strains of rats were used.

TABLE 1. CHANGES IN WEIGHT OF BODY AND ADRENAL GLANDS DURING ENTIRE EXPERIMENT

GROUP	NO. RATS	MEAN CHANGE BODY WT. (%)	MEAN ADRENAL WT. ¹
1 Ovar. ² -oil.....	13	+18.4 ± 4.3 ³	.273 ± .038
2 Ovar.-estrogen.....	9	+.66 ± 1.5	.333 ± .070
3 Ovar.-adr. ⁴ -oil.....	16	+.20 ± 5.8	
4 Ovar.-adr.-estrogen.....	12	-5.8 ± 2.7	

¹ Ratio: Adrenal weight (mg.)/body weight (gm.).
error of the mean. ⁴ Adr. = adrenalectomy.

² Ovar. = ovariectomy. ³ Standard

In this experiment, the ratio of adrenal weight/body weight was much lower in the Wistar as compared with the Long-Evans strain.

The hypophyses were not weighed, but were observed at autopsy to be greatly enlarged in those rats which were treated with estrogen.

Gross Observations. The hair grew back on the rats of *group 1* in alternating areas, these patterns resembling those which have been described by others for normal rats. No significant modification of this mode of hair replacement occurred as a result of ovariectomy (fig. 1).

Continuation of the estrogen treatment after ovariectomy in *group 2* successfully maintained a pronounced retardation in the growth of hair. At the termination of the experiment, this suppression was practically complete in 90 per cent of the cases (fig. 2). If any growth did occur, the areas of replacement were small, the hair thin and frequently short and occasionally consisted of only scattered coarse hairs.

Previous reports that adrenalectomy in the rat leads to acceleration of hair growth were confirmed by the results obtained in *group 3*. Eighty-one per cent of these rats were growing hair more extensively at the time of autopsy than was true of any of the animals which were ovariectomized and treated with oil (fig. 3). The

remaining 19 per cent possessed a pelage which was comparable in extent of development with that of the animals in *group 1*.

In *group 4*, over-growth of hair was macroscopically visible in 10 of the 12 rats at autopsy 3 weeks after adrenalectomy (fig. 4) in spite of continuation of treatment with the estrogen after adrenalectomy. It was necessary to kill the remaining 2 rats 12 days post-operatively and although hair had not yet appeared on the surface of the skin, microscopic examination of the skin samples showed growth of hair to be underway as extensively as in the other individuals of this group. Thus, in all of the cases, estrogen failed to maintain a state of suppressed growth following excision of the adrenal glands. Furthermore, comparing the results in *groups 3* and *4*, it is ap-

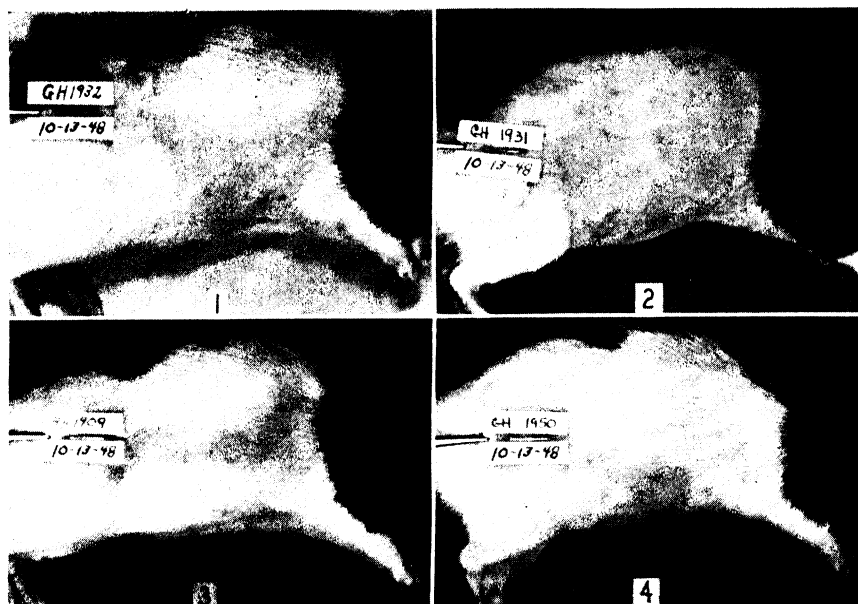


Fig. 1. OVARIECTOMIZED, oil treated. Fig. 2. OVARIECTOMIZED, estrogen-treated. Fig. 3. OVARIECTOMIZED, adrenalectomized, oil-treated. Fig. 4. OVARIECTOMIZED, adrenalectomized, estrogen-treated.

parent that growth of hair was more extensive in the latter group in spite of treatment with estrogen, although this difference cannot be considered as significant.

The dramatic nature of this response of the pilary system to adrenalectomy is made more impressive by the fact that it seemed to bear little or no relationship to the physical condition of the animal. Under treatment with estrogen several of the animals in *group 4* lost weight rapidly after the operation. Some were near death at autopsy. But regardless of their debilitated condition, hair was growing profusely over most of the area under observation. Such animals were in a state of poor nutrition and even though under-feeding has been shown to suppress the growth of hair (17), still the combined inhibiting action of reduced food intake and estrogen treatment failed to maintain a state of retardation in the growth of hair in the absence of the adrenal glands.

Microscopic Observations. Since two other proliferating epithelial structures exist in the skin, namely, the epidermis and sebaceous glands, it is of interest to discover whether or not parallel modifications might occur in their rate of growth after adrenalectomy and/or estrogen treatment.

The epidermis did not show evidence of release from inhibition following adrenalectomy combined with ovariectomy since its thickness was approximately within the normal range. Likewise, insofar as could be determined, there resulted no significant increase in number or size of the sebaceous glands.

After estrogen treatment (*group 2*) some thinning of the epidermis was observed confirming the previous observations of Hooker and Pfeiffer (5). Also, some atrophy and an occasional reduction in number of the sebaceous glands occurred but this was by no means a consistent finding. It could not be shown from our observations that adrenalectomy reversed this trend in the case of either epidermis or sebaceous glands.

DISCUSSION

Since considerable evidence shows that estrogens may stimulate the adrenal cortex by way of the anterior hypophysis (20), the possibility must be considered that estrogens might inhibit growth, at least partially, by stimulating the anterior hypophysis. The resulting increased liberation of adrenocorticotropin could in turn induce accelerated secretion of adrenocortical steroids, which presumably would be chiefly of the C-11 oxygenated type. Adrenocorticotropin, as well as extracts and pure compounds of the adrenal cortex, have been shown to retard growth of the body as a whole (21-23) and of certain proliferating tissues within it (24, 25). The demonstration that under the conditions of this experiment, estrogen could not inhibit growth of hair after adrenalectomy is at least compatible with this hypothesis.

This concept touches upon one of the most debated questions in endocrinology; namely, do estrogens stimulate or inhibit the anterior hypophysis? Estrogens induce a cytological picture in the hypophysis, which without doubt is one of stimulation (26, 27). Nevertheless, most physiologists and clinicians continue to regard estrogens as being effective inhibitors of pituitary secretion. One reason for the latter position is the fact that administration of estrogens may retard the rate of body growth, and several investigators have explained this effect on the basis of suppressed secretion of growth hormone by the anterior hypophysis (1, 28, 29). In fact, it has been found that concurrent injections of growth hormone could override the growth-inhibiting action of certain estrogens (30-32). However, it must be pointed out that a clear antagonism between purified growth and adrenocorticotropin preparations has been demonstrated in the hypophysectomized rat (33). Thus, injection of growth hormone into animals also receiving estrogen may merely provide enough growth hormone to counter-balance the effect of an elevated level of endogenous adrenocorticotropin brought about by the stimulating action of estrogen on the anterior hypophysis.

Since the rats in this experiment ate *ad libitum*, one must ask to what extent reduction of food intake modified the effects observed after estrogen treatment or adrenalectomy. It is well-known that administration of estrogen at high levels causes reduced intake of food; underfeeding, likewise, retards the growth of hair (17). However, Hooker and Pfeiffer (5) secured evidence which indicates that treatment with

estrogen slows the growth of hair even though food intake may not be reduced. As far as the post-adrenalectomy effects are concerned, there is at present little information available which would indicate that inadequate nutrition might have the reverse effect of accelerating the rate of growth or of making it impossible for estrogen to exert its usual inhibitory effect.

There remains the possibility that the adrenal cortex does not mediate the action of estrogen but rather that its secretions facilitate either directly or indirectly the action of estrogen in the peripheral tissues. Thus, Ingle (34) has demonstrated that adrenocortical substances potentiate the diabetogenic action of stilbestrol in this manner. This question is not answered by the experiment reported herein. Thus, the precise manner in which adrenalectomy counteracts the growth-inhibiting action of estrogen is not apparent at the present time.

If adrenalectomy results in removal of growth-inhibitors from the blood stream, this operation might be expected to cause an over-growth of epidermis and sebaceous glands. No satisfactory explanation is apparent for our failure to observe such changes. However, it might be related to a slower rate of cellular proliferation in these structures, as well as to the probable temporary character of the acceleration in rate of growth which occurs after adrenalectomy.

This study which again emphasizes the growth-inhibiting capacity of adrenocortical steroids bears on the recent report of Szego and Roberts (35) which showed that the hypophysis acting through the adrenal cortex antagonizes the stimulating action of estrogen in the uterus. Among other effects, estrogen promotes growth of the uterus. Thus, it is possible that in the experiments of Szego and Roberts the damping action of adrenocortical secretions on estrogenic stimulation of the uterus may have been essentially a growth-inhibiting phenomenon.

SUMMARY

Adult female rats were divided into the following groups: 1) ovariectomized, oil-treated; 2) ovariectomized, estrogen-treated; 3) adrenalectomized, ovariectomized, oil-treated, and 4) adrenalectomized, ovariectomized, estrogen-treated. Prior to the operations, alpha-estradiol dipropionate was injected into the rats of groups 2 and 4 at a daily dose of 20 to 40 μ g. until regrowth of hair was suppressed for 3 successive weeks. It was found that although estrogen maintained this inhibition subsequent to ovariectomy, after adrenalectomy it failed to prevent the acceleration in rate of hair growth which normally follows this operation.

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EXCRETION AND REABSORPTION OF SODIUM AND WATER IN THE ADRENALECTOMIZED DOG¹

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THE adrenalectomized animal and the patient with severe Addison's disease exhibit no less than three well recognized renal functional deficits: 1) an inability to limit sodium output to intake when normal or subnormal quantities of salt are ingested, with the consequence that body stores of sodium suffer progressive depletion (1-3); 2) a relative inability to excrete such metabolic waste products as nitrogen, potassium, phosphate and sulfate, which eventuates in the uremia of terminal adrenal insufficiency (4-7); and 3) an inability to eliminate ingested water with usual rapidity, a fact which renders the organism susceptible to water intoxication (8, 9).

Although it is generally accepted that failure to conserve sodium in adrenal insufficiency is due to a lack of normal hormonal regulation of tubular function, there is little agreement as to the nature of the renal defect. According to one view (10), it results from a reduced capacity of the tubular cells to perform the osmotic work involved in the nearly complete absorption of sodium from the glomerular filtrate, a condition which must be satisfied if sodium balance is to be maintained on a low salt intake. Another concept ascribes sodium loss to inability of the kidney to form ammonia (11), with the consequent draining of fixed base from the body to neutralize metabolic acid. According to a third view (12), increased sodium excretion is the passive consequence of a diuresis due to elevated plasma potassium. Finally it has been suggested (13) that there exists normally an hormonal balance between the antidiuretic, chloruretic and natriuretic actions of circulating pitressin and the diuretic and chloride and sodium conserving actions of the adrenal cortical principle. Disturbance of this balance by removal of the adrenal glands leads to urinary loss of chloride and sodium and inability to excrete water promptly.

Whatever its cause, loss of sodium and nearly equivalent quantities of water lead to dehydration, oligemia, reduction in glomerular filtration rate, and eventually to uremia (14, 15). Which of the several excretory deficiencies exhibited in adrenal insufficiency are secondary to circulatory inadequacy, and which are primary and depend specifically on increased tubular absorption cannot be stated with certainty, for most functional studies have been made on animals in a state of incipient circulatory collapse, or on patients with Addison's disease of relatively long standing and unknown prior renal capacity.

The present study was undertaken to assess renal function, especially with regard to the absorption and excretion of sodium, in adrenalectomized dogs maintained with adequate circulatory function by means of a high salt intake and intermittent treatment with desoxycorticosterone and adrenal cortical extract. It was found under such conditions that the rate of glomerular filtration remained within

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normal limits for some 2 or 3 months following adrenalectomy in the 5 dogs studied. Obviously in these animals delayed excretion of water and retention of potassium must be consequences of increased tubular reabsorption, not of reduced filtration from the plasma. Although at normal or subnormal plasma levels the reabsorption of sodium from the glomerular filtrate was less complete following adrenalectomy than in the normal state, at elevated plasma levels the reabsorption of sodium was more complete. Adrenalectomized animals therefore exhibited a reduced capacity to excrete sodium under high salt load analogous to their reduced capacity to excrete water under high water load. Despite this reduced excretory capacity for sodium, the urine formed during salt loading was hypertonic, and the osmotic work involved in its formation often exceeded that observed under similar conditions in intact animals. Our experiments suggest that sodium loss at normal and subnormal plasma levels, relative inability to excrete water and salt under load, and formation of a highly hypertonic urine may depend upon a common factor of overproduction of, decreased destruction of, or hypersensitivity of the kidney to antidiuretic hormone.

METHODS

Experiments were performed on female mongrel dogs loosely restrained on a comfortable animal board. For the majority of experiments (cf. tables 1, 2, 4, and figs. 1, 2) the following standard procedure was adopted. The animal was fasted for 14 to 18 hrs. and 500 cc. of 0.9 per cent saline was administered per os approximately 1 hour prior to the experiment. Creatinine, in amounts sufficient to insure a plasma level of 20 to 30 mg. per cent and 50 m.U. of pitressin² were administered in a priming dose. A sustaining infusion of creatinine in 5 per cent glucose containing 50 m.U. of pitressin per hour was given at a rate of 2.5 cc. per minute during 2 control periods. The sustaining infusion was then changed to 2.5 or 3.5 per cent NaCl containing 50 m.U. of pitressin per hour and administered at a rate of 10 cc. per minute. Because of the relative fragility of the adrenalectomized dog, it was necessary to limit the infusion in most instances to 2.5 per cent NaCl.

After a variable number of experiments in the normal state, each dog was adrenalectomized in 2 stages, separated by one week. In the interval between stages, immediately postoperatively, and in intervals between experiments the animals were maintained on minimal daily maintenance doses of desoxycorticosterone³ (2.5 mg.) in oil, and allowed free choice of saline and water in their cages. Whenever they refused to eat, they were treated with whole adrenal cortical extract. For 3 to 5 days preceding an experiment all supportive measures other than free access to saline were withdrawn. Four of the 5 animals eventually died some hours following experiments in which hypertonic saline was infused. The remaining animal

² Since the subsequent infusion of hypertonic saline would presumably stimulate the liberation of endogenous pitressin (16), it was felt that excessive variations in antidiuretic hormone activity throughout the course of the experiment could be avoided to some extent by administering these quantities from the start.

³ We are indebted to the Schering Corp. for the liberal supply of desoxycorticosterone used in these experiments.

died suddenly without apparent cause.⁴ All were autopsied and in none was any residual cortical tissue found.

Chemical methods and procedures have been described in other communications (17, 18). All sodium determinations except those reported in table 3 were made by the Butler and Tuthill (19) gravimetric method. Sodium and potassium values presented in this latter table were determined with an internal standard flame photometer accurate within limits of ± 2 per cent.

RESULTS

Glomerular Filtration Rate in the Hydrated Adrenalectomized Dog. In a series of 5 dogs adequately hydrated by allowing free access to salt and water at all times and given 500 cc. of saline per os prior to an experiment, glomerular filtration rate was essentially unchanged from the control preoperative level for periods up to 3 months following adrenalectomy. In table 1 are presented the filtration rates determined in the

TABLE 1. RATES OF GLOMERULAR FILTRATION OF 5 DOGS IMMEDIATELY BEFORE AND FROM 8 TO 10 DAYS AFTER ADRENALECTOMY

DOG NO.	GLOM. FILT. RATE cc./min.		DAYS POST OPERATIVE
	Control	Adrenalectomized	
1	61.8	61.9	8
2	34.6	31.2	8
3	53.9	48.7	10
4	63.3	59.7	8
5	57.6	61.0	8

last experiment preceding the initial stage of adrenalectomy and in the first experiment following the final operation. Identical procedures were followed in the pre- and postoperative experiments. In no instance was a significant change in filtration rate produced by adrenalectomy.

In table 2 is presented a more extensive series of observations on one dog which extended over a period of 7 months. Up to $2\frac{1}{2}$ months following adrenalectomy, i.e. through February 24, 1948, no significant change in filtration rate occurred. Whether the observations on March 11, March 23 and March 30 represent true decreases in filtration rate is debatable in view of the similarly low value on February 3, 1948. However, from April 13 to June 24 a real decrease in filtration rate is apparent. During this interval the animal was in excellent condition and exhibited no obvious signs of adrenal insufficiency. These observations are in agreement with results previously reported on rats which indicate that adrenalectomy per se does not alter glomerular filtration rate in well hydrated animals studied within a few weeks of operation (20, 21). Although prolonged observations were made only upon a single animal it would appear that in the chronically adrenalectomized dog as in patients with Addison's disease (22, 23), glomerular filtration rate may decrease to subnormal levels despite adequate hydration.

Acute Changes in Renal Function in the Adrenalectomized Dog Following Replacement

⁴ This animal had been subjected to a trial withdrawal of supportive therapy some 3 weeks prior to death and exhibited changes in plasma composition typical of adrenal insufficiency.

Therapy. Experiments presented in table 3 not only illustrate the quantitatively small deviations in renal function which account for electrolyte imbalance in the adrenalectomized animal, but likewise demonstrate the rapidity with which some of them can be corrected by the administration of whole adrenal cortical extract and desoxycorticosterone acetate. The procedure followed in these experiments deviated from that in the ones just described, both in the initial preparation of the animal and in the infusions administered. Maintenance doses of desoxycorticosterone were discontinued 4 days prior to each experiment. For 3 days the animal was maintained on 0.6 per cent sodium chloride *ad lib.* and for the final day on tap water. No saline was given at the start of the experiment and creatinine and paraaminohippurate in 3 per cent glucose was infused at 1 cc. per minute throughout the experiment. No pitressin was given.

In the 4 control periods of each experiment some 97.5 to 98 per cent of the filtered sodium was reabsorbed from the glomerular filtrate. The 2 to 2.5 per cent excreted could, if continued, account for the loss each day of the sodium contained

TABLE 2. RATES OF GLOMERULAR FILTRATION OF DOG 1 DETERMINED AT INTERVALS OVER A PERIOD OF 7 MONTHS

DATE	GLOM. FILT. RATE cc./min.	DATE	GLOM. FILT. RATE cc./min.
12/ 4/47	61.8	3/11/48	54.4
12/ 6/47	1st stage adrenalectomy	3/23/48	52.1
12/13/47	2nd stage adrenalectomy	3/30/48	50.6
12/22/47	61.9	4/13/48	36.5
1/27/47	78.3	4/29/48	21.4
2/ 3/48	53.8	6/11/48	21.3
2/10/48	68.8	6/18/48	25.5 ¹
2/17/48	63.2	6/24/48	29.8 ¹
2/24/48	73.0		

¹ No saline administered prior to these experiments.

in approximately one liter of extracellular fluid. Although plasma sodium was normal because of the relatively short period of withdrawal of all supportive measures, plasma potassium was significantly elevated. The absorption of some 85 per cent of the filtered potassium is somewhat greater than that observed in the normal dog, and no doubt accounts for the elevated plasma level. The administration of 20 ml. of whole adrenal cortical extract (Upjohn) reduced the excretion of sodium from an average of 73 to 4 μ Eq. per minute within a period of 80 minutes. This reduction in excretion was brought about by an increase in the quantity reabsorbed to 99.9 per cent of that filtered. Conversely potassium excretion increased from an average of 24 to 77 μ Eq. per minute at which time less than half of that filtered was reabsorbed. In the second experiment similar effects were obtained following the intravenous administration of 5 mg. of desoxycorticosterone emulsified in sesame oil and water. However, the time courses of the urinary electrolyte changes were somewhat different, in that the reduction in sodium excretion and especially the increase in potassium excretion occurred more rapidly in the latter experiment. The data of the two experiments emphasize that both agents act primarily upon the absorption of sodium and potassium, and affect the absorption of chloride indirectly. This is especially

evident in the second experiment in which the prompt increase in potassium excretion more than compensated for the initial slight decrease in sodium excretion. Accordingly urinary chloride first rose sharply above and then fell gradually below the control level due first to the outpouring of potassium and then to the increased reabsorption of sodium. This latter experiment likewise illustrates the fact that the changes in reabsorption of potassium and sodium may have independent time courses,

TABLE 3. IMMEDIATE EFFECTS OF ADMINISTRATION OF ADRENAL CORTICAL EXTRACT AND DESOXYCORTICOSTERONE ON RENAL FUNCTION OF THE ADRENALECTOMIZED DOG

URINE FLOW	GLOM. FILT. RATE	RENAL PLASMA FLOW	PLASMA			EXCRETED			REABSORBED ¹		
			Na	K	Cl	Na	K	Cl	Na	K	Cl
			mEq/l.			μEq/min.			% filtered		
<i>Dog 1; Adrenalectomized</i>											
1.35	27.9	91.6	146.5	5.80	116.7	64.0	19.0	89.0	98.35	87.63	97.40
1.40	26.1	85.8	146.1	5.83	114.3	79.0	23.0	77.0	97.88	84.10	97.55
1.50	24.3	80.6	143.5	5.74	113.0	76.0	32.0	70.0	97.71	75.87	97.57
1.40	25.8	85.0	145.2	5.62	116.0	72.0	22.0	66.0	97.98	83.04	97.90
<i>20 cc. whole adrenal cortical extract intravenously</i>											
0.64	27.7	91.0	145.0	5.26	114.6	79.0	27.0	77.0	97.93	80.50	97.69
0.48	25.5	84.8	142.9	4.95	115.3	75.0	31.0	84.0	97.83	74.15	97.28
0.40	28.8	96.0	143.0	5.16	115.2	52.0	54.0	58.0	98.67	61.90	98.33
0.65	27.4	98.8	144.5	4.85	114.1	4.3	51.0	41.0	99.89	59.58	98.75
1.27	30.3	112.0	144.7	4.92	113.6	6.1	64.0	43.0	99.85	54.78	98.81
1.50	32.4	122.6	145.2	4.66	114.1	3.2	77.0	50.0	99.93	46.30	98.71
<i>Dog 1; Adrenalectomized</i>											
0.70	29.2	91.5	143.5	5.94	114.6	107.0	25.0	91.0	97.31	84.83	97.54
0.70	29.1	87.8	142.2	5.86	114.4	81.0	23.0	75.0	97.94	85.79	97.86
0.60	28.6	84.4	143.2	5.54	113.6	88.0	25.0	80.0	97.74	83.38	97.66
0.55	32.3	95.7	140.8	5.55	114.6	96.0	31.0	86.0	97.78	81.80	97.79
<i>5 mg. desoxycorticosterone acetate intravenously</i>											
0.60	33.2	102.0	143.2	5.21	114.0	80.0	93.0	160.0	98.23	43.22	95.97
0.58	31.8	98.0	141.4	5.12	114.0	63.0	63.0	106.7	98.58	59.23	97.20
0.35	31.5	105.6	141.3	4.78	113.2	25.0	46.0	61.0	99.41	67.80	98.37
0.33	35.4	123.3	141.3	4.75	112.5	6.0	50.0	52.0	99.87	68.75	98.76
0.45	35.9	132.5	141.3	4.47	112.5	5.0		57.0	99.90		98.66
0.35	35.0	121.0	141.3	4.66	112.3	5.0	51.0	43.0	99.89	67.10	98.96

¹ The quantities filtered have been calculated with assumed Donnan factors of 0.95 for sodium and potassium and 1.05 for chloride.

and therefore that increased reabsorption of one ion species cannot be reciprocally and causally related to decreased reabsorption of the other.⁵

Both renal plasma flow and glomerular filtration rate increased following adrenal cortical extract and desoxycorticosterone, a fact which has been previously noted in the adrenalectomized animal (24). However, the changes in cation excretion are clearly independent of the changes in filtration rate, i.e., sodium excretion fell despite

⁵ Such independence has been observed in a number of subsequent experiments in which desoxycorticosterone has been administered intravenously.

an increased delivery of filtrate into the tubules, and potassium excretion increased far more than can be ascribed to the increase in filtration rate. Furthermore it is apparent that neither agent increased blood flow or filtration rate to anything like the normal preoperative value for the animal (62 cc. per min.).

Comparison of the Reabsorption and Excretion of Sodium, Chloride, and Water by the Normal and Adrenalectomized Dog under Salt Load. In table 4 are presented 3 experi-

TABLE 4. EXCRETION AND REABSORPTION OF SALT AND WATER UNDER LOAD BY THE NORMAL DOG AND BY THE ADRENALECTOMIZED DOG. ALL EXPERIMENTS ON DOG I

URINE FLOW	GLOM. FILT. RATE	PLASMA		EXCRETED		REABSORBED ¹						
		Na	Cl	Na	Cl	Na	Cl	Na	Cl	Na	Cl	H ₂ O
cc/min.		mEq/l.		mEq/min.		mEq/min.		mEq/100 cc. filtrate		% filtered		
Normal												
1.35	62.7	145.5	114.6	0.27	0.30	8.40	7.25	13.37	11.55	96.9	96.1	97.6
1.15	66.4	145.0	115.8	0.27	0.31	8.87	7.76	13.37	11.68	97.0	96.2	98.1
Infuse 3.5% NaCl at 10 cc./min.												
7.95	82.1	145.9	120.7	1.57	1.62	9.80	8.79	11.93	10.71	86.2	84.5	89.6
13.80	75.8	147.8	127.5	2.32	2.32	8.32	7.83	10.95	10.33	78.2	77.1	80.4
19.70	75.5	150.4	130.1	3.10	3.16	7.68	7.15	10.16	9.47	71.2	69.4	72.0
21.00	84.6	158.9	136.1	3.32	3.41	9.45	8.68	11.15	10.26	74.0	71.8	73.3
Adrenalectomized												
0.80	63.3	139.6	114.2	0.11	0.16	8.28	7.43	13.09	11.74	98.7	97.9	98.6
0.60	60.6	140.8	113.7	0.13	0.15	7.97	7.09	13.16	11.70	98.4	97.9	99.0
Infuse 3.5% NaCl at 10 ml./min.												
1.70	60.8	143.6	119.0	0.31	0.41	7.98	7.19	13.14	11.82	96.2	94.6	97.1
4.00	65.3	151.1	126.3	0.69	0.78	8.68	7.88	13.30	12.06	92.6	91.0	93.4
5.40	63.3	155.9	132.2	0.95	1.03	8.42	7.76	13.30	12.25	89.8	88.3	91.0
6.00	66.0	160.9	139.1	1.02	1.07	9.06	8.57	13.72	12.99	89.8	88.9	90.2
Adrenalectomized												
0.50	58.6	144.0	119.7	0.10	0.14	7.92	7.23	13.51	12.34	98.8	98.1	99.1
0.65	61.7	143.8	120.3	0.11	0.16	8.32	7.64	13.49	12.37	98.7	98.0	98.8
Infuse 2.5% NaCl at 10 cc./min.												
1.10	71.7	144.6	126.0	0.30	0.36	9.55	9.14	13.30	12.72	97.0	96.2	98.4
2.40	78.0	146.3	129.4	0.67	0.66	10.17	9.94	13.05	12.72	93.9	93.8	96.7
3.55	77.4	151.8	133.5	0.98	0.95	10.17	9.90	13.19	12.80	91.2	91.2	95.0

¹ The quantities filtered have been calculated with assumed Donnan factors of 0.95 for sodium and 1.05 for chloride and a water content of plasma of 94%.

ments, the first performed before adrenalectomy, the second and third after adrenalectomy, in which the reabsorption and excretion of sodium, chloride and water were compared. In each experiment, the initial two clearance determinations were made prior to, and the remainder during, the infusion of hypertonic saline. The rates of excretion of sodium and chloride during the initial 2 periods were relatively high before as well as after adrenalectomy, for 500 cc. of 0.9 per cent saline were administered per os at the start of each experiment.

In the normal dog the response to the infusion of hypertonic saline was invariably

a marked increase in glomerular filtration rate and in urine flow. In the adrenalectomized animal the increase in filtration rate was inconstant. Thus in *experiment 2* little change in filtration rate was observed; in *experiment 3* a very significant increase occurred. But independent of whether or not filtration rate increased, urine flow of the adrenalectomized animal never approached that of the normal animal at comparable plasma sodium levels. Thus in *experiment 2*, following adrenalectomy urine flow reached a value of 6 cc. per minute, whereas in *experiment 1*, performed prior to adrenalectomy, urine flow at a somewhat lower plasma sodium level reached 21 cc. per minute. In *experiment 3*, filtration rate increased to approximately the same degree as in *experiment 1*, yet the increase in urine flow was insignificant. Very obviously the oliguria of the adrenalectomized animal under salt load as under water load is not related to decreased filtration but rather to increased absorption of water.

The rates of excretion of sodium and chloride increased in all experiments, roughly mirroring the changes in plasma concentration. However, the magnitudes of these increases were significantly greater in the normal than in the adrenalectomized animal. The basis of this relative deficiency in excretion of both salt and water by the adrenalectomized animal is evident in the alterations in tubular reabsorptive activity which followed operation, and which are illustrated in table 4. In the experiment on the normal animal, the absolute rates of reabsorption of sodium and chloride (expressed in mEq. per min.) increased moderately as plasma levels were raised. However per 100 cc. of filtrate, reabsorption decreased sharply in consequence of the marked increase in filtration rate. In the first experiment following adrenalectomy the absolute rates of reabsorption of sodium and chloride likewise increased moderately, in the second the increases were considerable. However, in contrast to the result obtained in the intact animal, reabsorption per 100 cc. of filtrate either remained constant or actually increased slightly in the operated animal.

When reabsorption is expressed in terms of per cent of the quantity filtered, the basis for the diminished excretory capacity of the adrenalectomized animal is even more clearly evident. The normal animal, initially absorbing from 96 to 98 per cent of the filtered sodium⁶, chloride and water, absorbed only 72 to 74 per cent when loaded by an infusion of hypertonic saline. Under a similar salt load the adrenalectomized animal continued to absorb 89 per cent or more of the filtered ions and water.

Figures 1 and 2 summarize the results of all experiments performed in the manner just described. In figure 1 the per cent of filtered sodium reabsorbed and the per cent excreted are plotted against plasma sodium concentration. It is apparent that adrenalectomized animals reabsorb a greater percentage and excrete a lesser percentage of the filtered sodium than do intact animals at plasma concentrations above a level of approximately 145 mEq. per liter. At lower plasma concentrations the reverse is true; i.e., normal animals reabsorb a greater proportion of the filtered sodium than do adrenalectomized animals. The relationship for chloride is qualitatively so similar to that for sodium under the conditions of these experiments, that presentation of the data is unnecessary.

In figure 2 the per cent of filtered water absorbed and the per cent excreted are

⁶ This initial high rate of sodium excretion in the normal animal is the consequence of the administration of 500 cc. of saline at the start of the experiment.

likewise plotted against plasma sodium concentration. Under equivalent conditions of salt load, it is apparent that adrenalectomized animals reabsorb a greater percentage and excrete a lesser percentage of filtered water than do normal animals. However, at normal plasma sodium levels little difference in water excretion is evident in the two groups. This latter finding is obviously conditioned by our experimental procedure of administering saline per os at the start, and by infusing pitressin throughout the entire course of each experiment.

Effect of Adrenalectomy on Capacity of the Kidney to Perform Osmotic Work. In a majority of the experiments in which hypertonic saline was infused, the deficiency in excretion of water by the adrenalectomized animal was disproportionately greater than the deficiency in excretion of salt. Accordingly, urinary concentrations of sodium and chloride in operated animals usually exceeded those in intact animals at equiv-

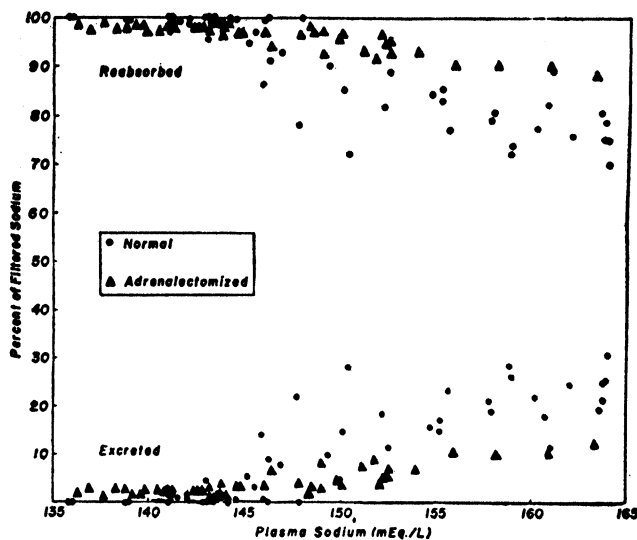


Fig. 1. REABSORPTION AND EXCRETION of sodium by the intact and adrenalectomized dog at normal and elevated plasma sodium levels

alent plasma levels. At the highest plasma sodium concentrations attained with infusions of hypertonic saline, normal animals excreted urine in large volume with essentially the same sodium and chloride contents as the plasma. Osmotic work on sodium and chloride approached zero. In contrast, although less urine was excreted by adrenalectomized animals, urine/plasma concentration ratios for sodium and chloride often approached 2. Accordingly the osmotic work performed by the kidney of the adrenalectomized animal on these ions usually exceeded that of the normal animal.

Calculations of osmotic work⁷ in two representative experiments are presented in table 5. In the normal animal, work on sodium and chloride was greatest in the first period following salt infusion and amounted to 0.162 gm. cal/min. In subsequent

⁷ Osmotic work was calculated using the formula of Rapoport *et al.* (25): $W = RTV (U \ln U/P + P - U)$.

periods work decreased, for although urine flow increased greatly, urine salt concentration fell to approximate plasma concentration. Others have observed that the kidney of the normal subject does not efficiently concentrate sodium and chloride under high salt load (25). However, as is apparent in table 5, the kidney of the adrenalectomized animal performed progressively increasing amounts of work throughout the three periods of salt infusion, reaching a value of 0.202 gm. cal/minute. Although the calculated values for osmotic work on sodium and chloride do not represent the entire osmotic load borne by the kidney, it is probable that the total renal work of the adrenalectomized animal exceeded that of the normal animal. At least there is no evidence of any incapacity of the kidney of the adrenalectomized animal to perform osmotic work on these two ion species.

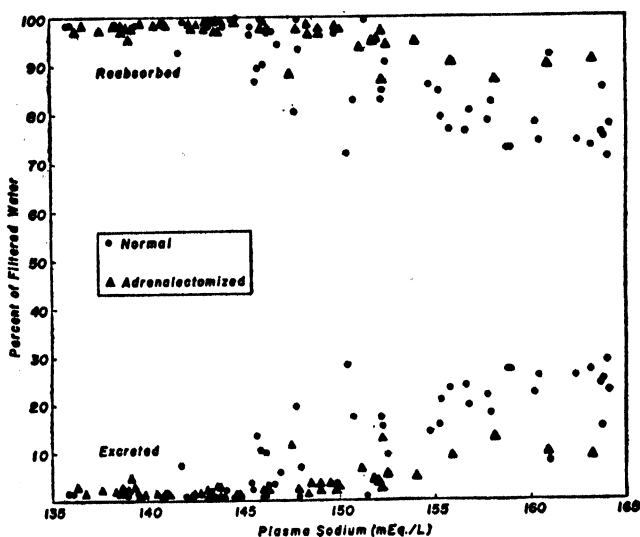


Fig. 2. REABSORPTION AND EXCRETION of water by the intact and adrenalectomized dog at normal and elevated plasma sodium levels

DISCUSSION

The relatively low volume and high salt content of the urine, and the very considerable osmotic work performed by the kidney of the adrenalectomized animal under conditions of salt loading suggest an exaggerated effect of posterior pituitary antidiuretic hormone. Following removal of the adrenal cortex, the natriuretic and antidiuretic actions of the posterior pituitary hormone are unopposed by the salt conserving and diuretic actions of the cortical hormones, leading to an apparent increase in antidiuretic hormone activity. Such a view was expressed a number of years ago by Silvette and Britton (13). In addition, the possibilities exist that *a*) the secretion of pitressin may be actually increased following adrenalectomy or *b*) there may be decreased destruction of antidiuretic hormone by the liver³ in the adrenalectomized animal.

³ Work in progress at the present time in the Zoology Department at Syracuse indicates that there is a decreased rate of pitressin destruction by the liver of adrenalectomized rats 5 days postoperatively.

tomized animal. The latter view is in accord with the observations of Birnie *et al.* (26) that there exists an increased concentration of antidiuretic hormone-like agent in the blood of adrenalectomized rats and that the adrenalectomized animal exhibits increased sensitivity to injected pitressin. Similar results have been obtained by Lloyd (personal communication) in patients with Addison's disease.

That the adrenalectomized animal and the patient with Addison's disease exhibit not only a reduced diuretic response to a standard water load (8, 9) but also a reduced ability to reabsorb sodium and chloride at normal plasma levels (15) is well recognized. The deficiency in capacity to excrete water results from excessive tubular reabsorption, for as previously shown in the rat (20, 21) and as demonstrated above

TABLE 5. COMPARISON OF OSMOTIC WORK PERFORMED ON SODIUM AND CHLORIDE BY THE KIDNEY OF THE NORMAL AND ADRENALECTOMIZED DOG. EXPERIMENTS 1 AND 3 OF TABLE 4 PRESENTED

URINE FLOW	SODIUM		CHLORIDE		OSMOTIC WORK ON		
	Plasma	Urine	Plasma	Urine	Sodium	Chloride	Sum
cc/min.	mEq/l.		mEq/l.		gm. cal/min.		
			<i>Normal</i>				
1.35	145.5	200	114.6	222	0.0076	0.0325	0.0401
1.15	145.0	235	115.8	269	0.0172	0.0518	0.0680
	<i>Infuse 3.5% NaCl at 10 ml/min.</i>						
7.95	145.9	198	120.7	204	0.0467	0.1152	0.1619
13.80	147.8	168	127.5	168	0.0102	0.0485	0.0587
19.70	150.4	157	130.1	160	0.0018	0.0376	0.0394
21.00	158.9	158	136.1	160	-0.0010	0.0243	0.0233
	<i>Adrenalectomized</i>						
0.50	144.0	200	119.7	280	0.0030	0.0242	0.0272
0.65	143.8	169	120.3	246	0.0012	0.0201	0.0213
	<i>Infuse 2.5% NaCl at 10 ml/min.</i>						
1.10	144.6	273	126.0	327	0.0304	0.0748	0.1052
2.40	146.3	279	129.4	275	0.0699	0.0904	0.1603
3.44	151.8	276	133.5	268	0.0886	0.1133	0.209

in the dog, glomerular filtration of water is within normal limits in the well hydrated animal, at least for a period of several months following removal of the adrenal glands. The increased natriuresis and chloruresis exhibited by the adrenalectomized animal is likewise independent of filtration rate and must represent decreased tubular absorption. That the overabsorption of water and the decreased absorption of sodium and chloride may be mediated by a posterior pituitary hormonal mechanism is suggested by the recent demonstration by Birnie *et al.* (26) of an increased concentration of a pitressin-like antidiuretic and chloruretic agent in the blood of adrenalectomized rats. This substance was not present in hypophysectomized and adrenalectomized rats and in all respects resembled pitressin.

It is likewise well established that pitressin not only stimulates the reabsorption of water by the renal tubule but also depresses the reabsorption of chloride and so-

dium (27). As shown in table 6, the infusion of as little as 50 m.U. of pitressin per hour into a normal animal may result in a nearly 10-fold increase in chloride excretion. If the highest rate of excretion shown in this experiment were maintained for 24 hours, it would result in the loss of 700 mEq. of chloride. This loss far exceeds that observed in the untreated adrenalectomized dog or patient with Addison's disease. It is reasonable to believe, therefore, that a relatively small excess of circulating pitressin could account at least in part for the negative chloride and sodium balance, the elevated urinary concentrations of these electrolytes, and the reduced diuretic response to a water load so commonly observed in adrenal insufficiency.

The relative incapacity of the adrenalectomized animal to excrete salt under high salt load, demonstrated in the experiments reported above, has been previously observed in patients with Addison's disease. Thus Greene *et al.* (28) and Reforzo-Membrives *et al.* (29) have shown that the sodium tolerance of patients with adrenal insufficiency is less than that of normal individuals, and when intake exceeds tolerance, edema to the point of anasarca develops.

TABLE 6. EFFECT OF INFUSING PITRESSIN ON EXCRETION OF CHLORIDE BY THE NORMAL DOG

URINE VOLUME		PLASMA CHLORIDE		URINE CHLORIDE	
cc/min.		mEq/l.		mEq/l.	mEq/min.
1.30		119.5		43.9	0.057
0.97		118.8		64.8	0.063
0.77		118.5		68.3	0.053
50 mU/hr. Pitressin					
1.40		121.6		158.8	0.222
2.28		120.4		201.0	0.458
2.55		118.0		191.5	0.488

It is generally accepted that pitressin has no antidiuretic effect when administered during the course of an osmotic diuresis induced by the administration of salt solutions. Furthermore because of its natriuretic and chloruretic effect, an exaggerated pitressin action should increase rather than decrease the capacity of the adrenalectomized animal to eliminate sodium and chloride under high salt load. Recent work in this laboratory has indicated that neither of the above statements can be accepted without reservation. Thus pitressin will often exert an antidiuretic effect during the diuresis induced by normal saline if the urinary concentration of sodium is less than 200 to 250 mEq. per liter and that of chloride is less than 300 to 350 mEq. per liter. Even after such urinary concentrations have been attained, moderate antiduresis may result from pitressin administration. Obviously curtailment of urine flow after maximum urine concentrations have been reached results in increased absorption of electrolyte. Thus the natriuretic and chloruretic actions of pitressin can be demonstrated only when the urinary concentrations of sodium and chloride are initially below their respective maxima. When at their maxima, pitressin does not increase ionic excretion; rather it decreases excretion in proportion to the reduction in urine volume. Lacking evidence to the contrary we interpret this increased ionic absorption as the passive consequence of increased absorption of water.

The demonstration that the kidney of the adrenalectomized animal concentrates

salt to a greater degree than does the kidney of the intact animal when a high salt load is imposed, suggests that a limitation of osmotic work capacity cannot explain the renal deficiencies of the operated animal. In experiment 3 of table 5 osmotic work was performed in concentrating sodium and chloride at a rate of 0.202 gm. cal. per minute. To absorb the sodium and chloride from 1 liter of urine in 24 hours, energy would have to be expended at a rate of only 0.108 gm. cal. per minute.⁹ It is true that the work is performed in the two instances in opposite directions, but were energy turnover alone involved, it would seem that directional orientation should not be a significant factor. We infer that inability to conserve sodium in the adrenalectomized animal is a specific renal defect imposed by hormonal imbalance, and is not a non-specific defect of energy metabolism.

The different time courses of the action of desoxycorticosterone on the absorption of sodium and on the elimination of potassium demonstrated in experiment 2 of table 3, suggest that the retention of potassium and the depletion of sodium which characterize the state of adrenal insufficiency are not causally interrelated. Although the hormone regulates the activity of both renal mechanisms, it can promote an immediate outpouring of potassium without simultaneously increasing sodium reabsorption to an appreciable degree. We therefore infer that the hormone acts on two functionally distinct tubular mechanisms in opposite fashion. Our observations confirm the view of Harrison and Darrow (24) that retention of potassium in the adrenalectomized animal is due specifically to tubular overabsorption and is not the result of reduced filtration of this ion from the plasma. In so far as our experiments have all been performed on well hydrated animals in good condition, they do not advance knowledge of renal function in terminal adrenal insufficiency.

SUMMARY

The rates of glomerular filtration of 5 well hydrated adrenalectomized dogs were maintained within normal limits for periods up to 3 months following operation. These animals exhibited a reduced capacity to eliminate sodium under high salt load, analogous to their reduced capacity to eliminate water under high water load. Reduction in sodium and water excretion under the conditions of these experiments is therefore due to increased tubular reabsorption rather than to reduced glomerular filtration. The low volume and high salt concentration of the urine and the very considerable osmotic work performed by the kidney of the adrenalectomized animal under salt load suggest an overproduction of, decreased destruction of, or hypersensitivity to antidiuretic hormone. Such a view is consistent with the known susceptibility of the adrenalectomized animal to water intoxication. In the light of the natriuresis and chloruresis produced in the normal animal by relatively small quantities of pitressin, this view is consistent with the negative sodium and chloride balances observed in untreated operated animals. Retention of potassium is a manifestation of specifically increased ionic reabsorption, and is not directly related to diminished sodium absorption.

⁹ The assumed values for plasma are $\text{Na} = 140 \text{ mEq/l.}$ and $\text{Cl} = 105 \text{ mEq/l.}$; those for urine are $\text{Na} = 1.40 \text{ mEq/l.}$ and $\text{Cl} = 1.05 \text{ mEq/l.}$

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MECHANISM OF AURICULAR FLUTTER AND FIBRILLATION

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TOPICAL administration of aconitine as a subepicardial injection of a dilute solution or an application of a few crystals on the epicardial surface of any part of the exposed auricle of a dog causes within a few minutes the appearance of a regular auricular tachycardia with rates of approximately 300 beats per minute. This tachycardia persists for an hour or more. Faradic stimulation of the right or left vagus nerve in the neck induces a remarkable increase of the auricular rate for the duration of the stimulation (7).

Often the injection of aconitine causes an auricular fibrillation to appear, particularly if a fresh solution is injected into the area of the sinus node. Cooling this small area with a thermode stops the fibrillation or flutter but both arrhythmias reappear within one or two seconds after interrupting the cooling (9). This result is not compatible with the existence of a circus movement. Cooling prolongs the refractory phase and therefore it may be possible that a circulating wave meets refractory tissue and comes to an end. It is, however, difficult to explain the reappearance of the fibrillation on interruption of the cooling unless we assume the existence of a focus of stimulus formation.

Against a circus movement and for the assumption of a center of rapid stimulus formation in auricular flutter and fibrillation also speak the results of stretch experiments during an auricular flutter elicited in the manner just discussed (10). Stretching invariably increases the rate and when a certain level (around 600 stimuli per minute) is reached auricular fibrillation appears. It is known that the mechanical stimulus of stretch leads to spontaneous rhythmic discharges in nerve or muscle or to an increase of rate of an existing stimulus formation (3). This phenomenon was even observed on bundles of specific tissues removed from the heart of the dog (4). Stretch does not, however, alter the speed of conduction. Were a circus movement present, the appearance of an increased rate of flutter and its change into fibrillation would require the assumption of increased speed of the circulating central wave.

These investigations were continued in order to accumulate further evidence that the aconitine tachycardia actually represents auricular flutter and not a tachycardia peculiar to this drug. It was also anticipated that a study of the onset of the tachycardia would develop further proof for the assumption of a rapid stimulus formation and against a circus movement.

METHOD

Adult dogs were used in all 27 experiments. During nembutal anesthesia and artificial respiration, the heart was exposed by removing the sternum and opening of the pericardium. The vagus nerves were severed in the neck. The regular tachycardia or auricular fibrillation was evoked by subepicardial injection of 0.05 cc. of a 0.05 per cent solution of aconitine crystals (Merck) at the area of the head of the sinus node at the angle between superior vena cava and right auricular appendix. The cooling was achieved with the aid of a small thermode containing ice-cold water. The peripheral end of the right or left vagus nerve was stimulated, if desired, by means of a Cambridge inductorium. The electrocardiograms were taken in lead II.

In most experiments reported here particular attention has been paid to the onset of the aconitine tachycardia. For this purpose simultaneous cooling of the site of the aconitine injection and faradic stimulation of the left vagus nerve were employed. Cooling ended the existing flutter and the simultaneous stimulation of the vagus caused A-V block so that the auricular activity could be studied unobscured by the ventricular complexes when the cooling was discontinued.

RESULTS

A few experiments which are typical for many others will be described.

Figure 1 was obtained in an experiment of May 6, 1947. Auricular fibrillation had appeared after the injection of aconitine. Cooling the area of injection stopped the fibrillation but at the end, just before its disappearance, flutter waves may be observed with a rate of 375 per minute. During the cooling, an auricular escape beat with a biphasic P wave was registered. After discontinuing the cooling, which lasted for about 3 seconds, 4 auricular extrasystoles arise, the last of which is blocked. Suddenly, a regular auricular tachycardia starts with a rate of 285; it gradually increases to 430 and then to 600 beats per minute and finally auricular fibrillation reappears. The form of the auricular waves after the cessation of auricular fibrillation and before its reappearance is identical.

From tracings like that in figure 1 it will appear that the regular tachycardia at the end of auricular fibrillation and the tachycardia just before it is reinstituted actually represent auricular flutter. With a slowing of the formation of stimuli during the cooling, fibrillation changes into flutter and the reverse takes place when, after cessation of the cooling, the rate of stimulus formation increases. The change from fibrillation to flutter by cooling of only a circumscribed small area is not compatible with a circus movement mechanism.

In the experiment of March 8, 1949 the administration of aconitine led to auricular flutter with an auricular rate of 290 and no A-V block (fig. 2). In this experiment simultaneous faradic stimulation of the left vagus nerve and cooling the head of the sinus node, where aconitine had been injected, were done. The beginning of the stimulation of the vagus is clearly visible by the distortion of the electrocardiogram by the faradic current; the end is marked by the disappearance of the distortion at the end of figure 2, shortly before the ventricles contract again. The cooling started just before the end of the auricular flutter and was discontinued about one second before the auricular activity was resumed. It lasted for only about 4 seconds.

The auricular rate increased during the stimulation of the vagus nerve to 330. Then the flutter disappeared abruptly due to the cooling. One normal sinus beat, apparently originating in a deeper non-cooled center of the sinus node, appeared followed by a blocked auricular wave. After a prolonged pause due to cooling, auricular activity started again with a gradual increase of rate from 158 to 300. At the end of the tracing the pattern of auricular flutter with full conduction to the ventricles and a rate of 300 is the same as at the beginning. The form of the auricular waves does not change.

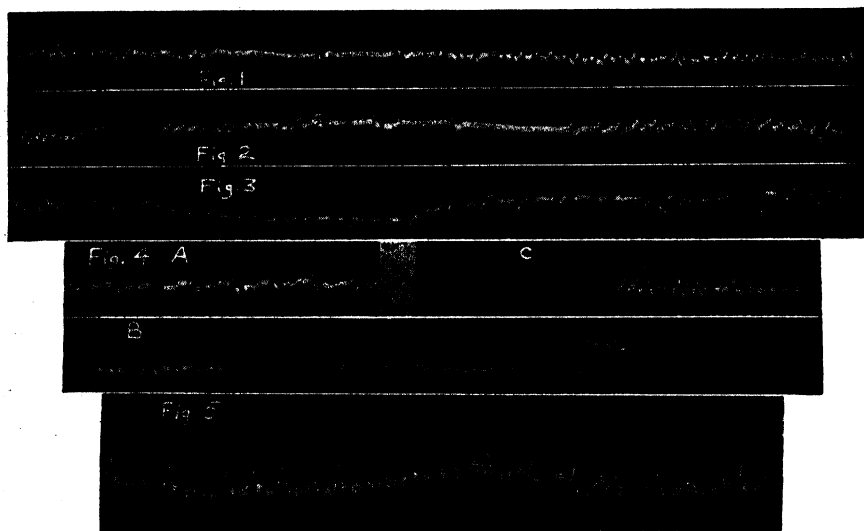


Fig. 1. COOLING OF SITE of injection of aconitine changes auricular fibrillation into a short flutter and then causes cardiac standstill. Cessation of cooling reestablishes flutter and with increase of auricular rate fibrillation appears.

Fig. 2. COOLING DURING VAGUS STIMULATION abolishes flutter; after interruption of cooling, flutter reappears with gradual increase of rate but no change of form of P waves.

Fig. 3. VAGUS STIMULATION during auricular flutter causes alternation of auricular diastole which cannot be explained by circus movement.

Fig. 4. A, SHOWS SINUS RHYTHM before and, B, sinus tachycardia after aconitine; vagus stimulation causes cardiac standstill. In the second half of B vagus stimulation during auricular flutter increases auricular rate. C is continuation of B.

Fig. 5. TOPICAL APPLICATION of potassium chloride changes auricular flutter into sinus rhythm.

The gradual increase of rate of the reappearing flutter without any change of the form of the P wave observed in this and all similar experiments speaks against the presence of a circus movement as the underlying mechanism. A continuous circus movement is impossible with the long diastoles between the first P waves after cessation of cooling. If one assumes that a path of different length is used with a change of rate of the flutter, the constancy of form of the auricular waves would remain unexplained. This experiment was repeated on 18 dogs, always with the same result.

Auricular flutter was elicited in the usual way in an experiment on December 7, 1948 (fig. 3). The flutter rate was 270 and every stimulus was conducted to the ventricles. The R waves of the ventricular complexes are so thin that they are not clearly

visible. With the beginning of the stimulation of the left vagus nerve the A-V conduction stopped immediately. The auricular activity continued but an alternation of successive auricular cycles appeared. The first auricular interval with the auricular wave buried in the T of the last ventricular complex measures 0.40 second; the following ones measure: 0.20, 0.24, 0.20, 0.23, 0.19, 0.24, 0.18, 0.30, 0.19, 0.28, 0.20, 0.24, 0.19, 0.33, 0.19, 0.28, 0.19, 0.28, 0.19, 0.33, 0.20, 0.33, 0.20, 0.34, 0.18, 0.45, 0.20, 0.28, 0.26, 0.19, 0.40, 0.20 and 0.35. With the discontinuation of the stimulation of the vagus the same picture reappears as at the beginning of figure 3.

A slight alternation of cycle length during the auricular flutter caused by aconitine was repeatedly seen without stimulation of the vagus. It is known to occur even in post-faradization flutter. Only in few experiments did it reach the degree seen in figure 3. The long diastoles between every second auricular wave rule out the existence of a circus movement; after a long standstill of the circulating wave its recurrence would be unexplainable. Slighter degrees of alternation of cycle length during an auricular tachycardia have been attributed to a circus movement in which alternately a path of different length is used (2). It has been shown, however, that such groups of 2 or more beats occur under conditions in which a circus movement is impossible (8). Multiple or repetitive discharges in groups may be caused by constant stimuli (1, 5); they have been seen even in small bundles of specific tissue (4). Another possibility, which is less probable, would be the presence of a regular rhythmical stimulus formation and periodically recurring blockade of the stimuli.

If we assume that flutter is due to a rapid stimulus formation in one focus, it is necessary to explain why the normal stimulus formation in the sinus node as well as the abnormal one during paroxysmal auricular tachycardia are often inhibited by the stimulation of the vagus nerves or vagal reflexes, while auricular flutter caused by faradization or by aconitine responds with an increase of rate. It was hoped that the study of the effects of faradic stimulation of the vagi at the beginning of aconitine tachycardia may throw some light on this problem. The results were in all experiments identical to that obtained on April 1, 1947 (fig. 5); it appears that we are actually dealing with two different types of stimulus formation.

The first tracing in the top row (fig. 4a) obtained before the injection of aconitine shows a regular sinus rhythm with a rate of 110 per minute. The first part of figure 4b shows a tachycardia of 200 beats per minute which had appeared about 70 seconds after application of a few aconitine crystals on the area of the head of the sinus node. Stimulation of the right vagus nerve leads to complete inhibition of the activity of the auricle and ventricles. With discontinuation of the stimulation of the vagus the sinus node resumes its activity and the rate gradually increases again to 200. Suddenly the rate rises to 310 (transition not registered) and vagus stimulation now causes a marked further increase of rate to 430 (second half of fig. 4b). Figure 4c in the top row represents the continuation of figure 4b. One finds that after interruption of the stimulation of the vagus the flutter rate fell quickly to 375 beats per minute. In other experiments the tachycardia caused by aconitine at first responded to vagus stimulation with standstill as shown in figure 4b and suddenly auricular fibrillation appeared.

From these experiments one may conclude that aconitine causes at first an

abnormal rapid stimulus formation comparable to the normal one in the sinus node or that seen in paroxysmal auricular tachycardia; suddenly another type of stimulus formation appears, which responds to vagus stimulation with an increase of rate.

If during the presence of auricular flutter or fibrillation solutions of quinidine or potassium were injected into the same area as the aconitine, sinus rhythm reappeared within a few seconds.

Figure 5 shows the change from auricular flutter with a rate of 250 into sinus rhythm after the injection of 0.1 cc. of a 1 per cent solution of potassium chloride into the focus of origin of the flutter; the latter was caused by aconitine injection into the same area.

DISCUSSION

The frequent transition of the rapid regular auricular tachycardia into auricular fibrillation and vice versa, particularly as seen in figure 1, demonstrates that we are really dealing with auricular flutter. In favor of this diagnosis also is the response to faradic stimulation of the vagus nerves with an increase of rate.

In addition to the effect of the cooling of the site of the injection of aconitine (fig. 1), the slow evolution of the flutter (fig. 2) and the appearance of long auricular pauses during the flutter (fig. 3) speak against circus movement as the underlying mechanism.

The hypothesis seems justified that we are dealing with a rapid stimulus formation in one center which with rates of over 600 causes in the dog the picture of auricular fibrillation because of the appearance of islands of refractory tissue in the auricles which force the excitation wave to take a circuitous path. In favor of this interpretation is also the effect of potassium or quinidine locally applied (fig. 5).

Aconitine causes, as figure 4 shows, at first the appearance of a heterotopic tachycardia which responds to stimulation of the vagus like a normal sinus rhythm or some paroxysmal auricular tachycardias observed in man with standstill or slowing. Suddenly another type of stimulus formation sets in which increases in rate when the vagus nerves are stimulated. One cannot help speculating that the stimuli which are inhibited by the vagi are rhythmically formed while the stimulus is continuous with the appearance of flutter. With the presence of a continuous stimulus it is conceivable that the auricular rate depends on the length of the refractory phase; its shortening by faradization of the vagus nerves should cause an increase of rate. It is known from the investigations of Lewis and his collaborators (6) that the conduction of stimuli within the auricles is not inhibited by the vagus.

SUMMARY AND CONCLUSIONS

The results of further studies of auricular flutter and fibrillation, caused by focal administration of aconitine on the dog's exposed auricle, are discussed.

It is demonstrated that auricular fibrillation can be transformed into auricular extrasystoles and flutter by slowing of the rate of stimulus formation with cooling the site of application of aconitine. Cessation of the cooling leads to reappearance of flutter; and when the rate of stimulus formation again increases, fibrillation appears.

During auricular flutter, stimulation of the vagus nerve and cooling of the site

of aconitine application are used simultaneously. The flutter is abolished by the cooling and when it reappears the rate slowly increases without any change of the form of the auricular waves. The long distance between the P waves cannot be explained by the presence of a circus mechanism.

Occasionally stimulation of the vagus nerves during the presence of auricular flutter causes long pauses to appear between the auricular waves; this speaks against the presence of a continuous circulating wave.

At the beginning of the action of aconitine a sinus tachycardia appears and is inhibited by stimulation of the vagus; later *suddenly* auricular flutter appears and its rate increases during stimulation of the vagi. From this it is concluded that we are dealing with two different types of stimulus formation. The hypothesis is advanced, that during auricular flutter there is a constant stimulus and the response of the auricle to this stimulus depends only on the length of the refractory phase.

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SOME FACTORS AFFECTING THE FOOD INTAKE OF NORMAL DOGS AND DOGS WITH ESOPHAGOSTOMY AND GASTRIC FISTULA

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EVIDENCE has been presented from this laboratory (1, 2) that the parenteral administration of glucose or protein hydrolysate to the rat and dog in amounts which form significant portions of the daily caloric intake does not produce a corresponding inhibition of food intake during a subsequent meal. When this is contrasted with the adjustment of food intake to bodily needs required for the constancy of body weight which the animal ordinarily manifests, it suggests that different factors may be operating in regulating the ingestion of food during any one meal from those involved in regulating food intake over longer periods of time.

As a man or animal ingests a meal, the further intake of food is progressively inhibited. The mechanism of this fact of daily experience is unknown. The evidence cited tends to indicate that supplying the cells of the organism with some of the products of normal digestion does not produce the inhibition which normally accompanies eating.

Accordingly, the present study was undertaken to investigate some of the factors involved in regulating the amount of food eaten by dogs at any one meal by studying 1) the effects of oral prefeeding of a portion of a meal upon subsequent food intake in intact dogs, 2) the effects of intragastric feeding upon food intake in dogs with gastric fistulas and 3) sham feeding and the effects of intragastric feeding on sham feeding in dogs with esophagostomies.

EFFECT OF ORAL SUPPLEMENT ON SUBSEQUENT FOOD INTAKE IN INTACT DOGS

Methods. Five healthy mongrel dogs were housed in an air-conditioned room in individual cages, and allowed to eat *ad libitum* of a complete dog food (Pard, regular, Swift) for 45 minutes daily. They were fed by the same individual and at the same time daily. There was free access to water at all times. Food intake was recorded daily and body weight weekly. After these became stabilized, a portion of his average daily intake was offered each dog 20 minutes before the regular feeding. This was done at irregular intervals and never more than twice weekly.

Results. These are summarized in table 1 and indicate that prefeeding with 200 grams of the regular diet inhibited the subsequent regular feeding by an approximately equivalent amount.

INTRAGASTRIC FEEDING IN GASTRIC FISTULA DOGS

Methods. Gastric fistulas were made in the flanks of 3 normal mongrel dogs, using a Thomas-type metal and hard rubber cannula, closed with a cork stopper.

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These dogs were housed in individual cages and the feeding and weighing routine was the same as described above. Within 2 months after operation, they were stabilized as to average daily food intake and body weight.

During the control periods the dogs were removed from their cages and placed in stocks for 20 minutes preceding each feeding period.

During test periods, varying amounts of the regular diet, or an equivalent volume of non-caloric bulk (Karaya gum), was introduced through the gastric fistula during the 20-minute period in the stocks. The dogs were then returned to their cages to be fed immediately or after 4 hours. Only one test was made on each dog weekly; the remaining days served as control days.

TABLE 1. EFFECT OF PREFEEDING A PORTION OF THE REGULAR DAILY MEAL TO INTACT DOGS

DOG NO.	PERIOD	NO. OF DAYS	AVERAGE FOOD INTAKE, GM.	GM. CHANGE FROM CONTROL PERIOD
15	Control	26	730	
	200 gm. Pard ¹	5	518	-212
16	Control	20	997	
	200 gm. Pard ¹	5	579	-418
17	Control	26	1272	
	200 gm. Pard ¹	5	1045	-237
18	Control	26	805	
	200 gm. Pard ¹	5	662	-143
19	Control	26	856	
	200 gm. Pard ¹	5	668	-188
Mean.....				-239

¹ Twenty minutes before regular feeding.

The results are summarized in table 2. It will be seen that volumes of 11 to 20 per cent of the average daily meal had no inhibitory effect on food intake; while volumes of 40 to 46 per cent of the average daily meal inhibited food intake 33 to 50 per cent, if the feeding followed immediately upon the intragastric feeding. Intragastric feeding 4 hours before the regular feeding had no such inhibitory effect. The results with non-caloric bulk were essentially the same.

SHAM-FEEDING EXPERIMENTS

Methods. Esophagostomies were performed according to the method of Dragstedt and Mullenix (3), with exteriorization of the esophagus in the neck in the first stage, and division by cauterization of the exteriorized esophagus in the second stage, 7 to 10 days later. Care was taken to avoid injury to the vagi. The dogs were fed and sham-fed upon a constant mixture of a complete dog food homogenized with milk in a Waring blender. This mixture was introduced into the stomach by tube through the distal esophageal opening (occasionally a small amount of cod liver oil was added

to the pabulum). On this regimen, 2 dogs maintained their body weight for over 3 months, and numerous other dogs were maintained in caloric equilibrium for shorter periods of time. Fluid needs were well satisfied for sham drinking rarely occurred. The length of sham feeding was taken as the time the dogs continuously sham-fed. A period of sham feeding was considered to be ended when interruption for more than 2 minutes occurred.

A. Sham-feeding time. Eight dogs with exteriorized but intact esophagus (stage one) were allowed to feed *ad lib.* three times daily for a period of 7 to 10 days. Average eating time was 2.5 minutes (range from 1.5 to 3.5 minutes). At the first regular feeding following division of the exteriorized esophagus, eating time was prolonged on the average of 14.1 minutes (range 7 to 31 minutes).

TABLE 2. EFFECT ON ORAL FOOD INTAKE OF INSTILLATION OF FOOD AND INERT SUBSTANCES INTO STOMACH OF DOGS WITH GASTRIC FISTULAS

DOG NO.	PERIOD	NO. OF DAYS	AVERAGE DAILY FOOD INTAKE, GM.	GM. CHANGE FROM CONTROL PERIOD
1	Control period	30	1034	
	200 gm. Pard, 20 min. before feeding	1	1185	+151
	450 gm. Pard, 20 min. before feeding	2	517	-517
	450 gm. Karaya, 20 min. before feeding	1	520	-514
	450 gm. Pard, 4 hr. before feeding	2	1175	+141
2	Control period	30	1754	
	200 gm. Pard, 20 min. before feeding	1	1820	+66
	700 gm. Pard, 20 min. before feeding	2	1173	-581
	700 gm. Karaya, 20 min. before feeding	1	1365	-389
	700 gm. Pard, 4 hr. before feeding	2	1760	+6
5	Control period	20	965	
	200 gm. Pard, 20 min. before feeding	1	920	-45
	450 gm. Pard, 20 min. before feeding	1	511	-454
	450 gm. Pard, 4 hr. before feeding	1	1237	+272

B. Effect of sham feeding on subsequent sham feeding. Dogs A and B were allowed to sham feed *ad lib.* after 18 hours of food deprivation, and then allowed to sham feed again at different intervals on separate days. The results appear in table 3. From this table it is apparent that in dog B there was an inhibitory effect which lasted 30 minutes, but that in both dogs inhibition was not present one hour after sham feeding.

C. Immediate effect of intragastric feeding on sham feeding. Dog A (17 lb.), and dog B (20 lb.) had been maintained on 350 to 400 cc. of pabulum respectively twice daily. Each had been allowed to sham feed *ad lib.* twice daily. Average sham-feeding time for dog A for 12 trials was 17 minutes (range 15 to 22 minutes) and for dog B for 12 trials was 17 minutes (range 14 to 25 minutes). An intragastric feeding of 350 cc. was then introduced in the course of 5 minutes just prior to a sham-feeding period. The average sham-feeding time was not significantly altered: dog A sham-fed 20.1

minutes in 8 trials (range 19 to 24 minutes), and *dog B*, 19.4 minutes in 8 trials (range 14 to 23 minutes).

D. *Effect of a larger intragastric feeding on sham feeding.* On three occasions 750 cc. of pabulum was introduced into the stomach of *dogs A* and *B* just prior to feeding. No significant inhibition of sham-feeding time as compared to the control period was observed. On two occasions, three intragastric feedings of 350 cc. each were introduced into the stomachs of both dogs at 2-hour intervals. Sham-feeding time following the third feeding was not altered as compared with control periods.

E. *Delayed effect of intragastric feeding on sham feeding.* In these 2 dogs the delayed effect of intragastric feeding was observed by noting sham-feeding time at intervals of 2, 3 and 4 hours after the intragastric feeding. The results given in table 4

TABLE 3. EFFECT OF INTERVAL SINCE PREVIOUS SHAM FEEDING ON SHAM-FEEDING TIME OF ESOPHAGOSTOMIZED DOGS

INTERVAL	CONTROL SHAM-FEEDING TIME		RE-SHAM-FEEDING TIME			
	18-hr. food deprivation (5 trials)		½ hr. (3 trials)		1 hr. (3 trials)	
	Av.	Range	Av.	Range	Av.	Range
	min.		min.		min.	
<i>Dog A</i>	22	(15-30)	26	(20-30)	20	(17-21)
<i>Dog B</i>	21	(19-22)	10	(5-15)	19	(19-22)

TABLE 4. EFFECT OF INTRAGASTRIC FEEDING AT VARIOUS INTERVALS BEFOREHAND ON DURATION OF SHAM FEEDING

DOG	INTERVAL AFTER INTRAGASTRIC FEEDING							
	Control (18 hr. after last feeding) (12 trials)		Two hr. (8 trials)		Three hr. (5 trials)		Four hr. (4 trials)	
	Time	Range	Time	Range	Time	Range	Time	Range
	min.		min.		min.		min.	
<i>A</i>	17.2	(15-22)	17.6	(15-20)	19.2	(20-30)	20	(18-24)
<i>B</i>	17.0	(14-25)	15.7	(14-18)	19.0	(17-22)	17	(15-22)

indicate that the introduction of a meal into the stomach did not alter the sham-feeding time 2 to 4 hours later, just as it had failed to inhibit sham feeding immediately after its introduction (*C*, above).

F. *Simulation of a real meal.* The attempt was made to simulate a real meal by allowing the esophagostomized dogs to sham-feed while the pabulum was simultaneously introduced into the stomach by a tube placed in the lower esophagostomy opening. The results on 5 dogs are tabulated in table 5. Control time is the sham-feeding time of the first period following division of the exteriorized esophagus. This was repeated in *dogs D* and *E* during 4 subsequent alternate sham-feeding periods (table 6).

The results presented in tables 5 and 6 show that introduction of food into the stomach during sham feeding significantly reduces sham-feeding time.

G. *Effect of gastric distention with a balloon on sham feeding.* The stomachs of

dogs *A* and *B* were distended by a soft rubber balloon inflated with air. Inhibition of sham feeding did not occur until 900 to 1000 cc. of air was introduced into the balloon, at a pressure of 3.5 to 4 cm. of mercury. This was accompanied by nausea, salivation and retching.

GENERAL DISCUSSION

The experiments detailed above indicate that the chewing and swallowing of food inhibits further intake whether or not food enters the stomach. Thus a period of sham feeding ceases even though the stomach is empty. When the food also enters the stomach, however, the inhibition of further intake occurs much more promptly. When, after esophagostomy, food is prevented from reaching the stomach, eating time is markedly prolonged. The attempts to simulate a real meal in esophagos-

TABLE 5. EFFECT OF SIMULTANEOUS INTRAGASTRIC FEEDING ON DURATION OF SHAM FEEDING

DOG	SHAM FEEDING CONTROL TIME	SHAM-FEEDING TIME DURING INTRAGASTRIC FEEDING	DOG	SHAM FEEDING CONTROL TIME	SHAM-FEEDING TIME DURING INTRAGASTRIC FEEDING
	min.	min.		min.	min.
<i>C</i>	11	5	<i>F</i>	11	6
<i>D</i>	17	10	<i>G</i>	10	5
<i>E</i>	31	11			

TABLE 6. EFFECT OF SIMULTANEOUS INTRAGASTRIC FEEDING ON DURATION OF SHAM FEEDING

DOG	CONTROL (4 TRIALS)		SHAM-FEEDING TIME DURING INTRAGASTRIC FEEDING (4 TRIALS)	
	Time	Range	Time	Range
	min.		min.	
<i>D</i>	23	(19-30)	11	(9-14)
<i>E</i>	20	(17-25)	11	(9-12)

tomized dogs indicate that gastric distention is operative only in close association with oral intake. The results of intragastric feeding in the gastric fistula dogs is in harmony with this observation, since in this instance also filling of the stomach occurs during oral ingestion, although partial filling preceded the beginning of eating.

Supplying part or all of the daily caloric need by intragastric feeding had no effect on the ingestion of the next regular meal in esophagostomized dogs; and supplying part of the daily caloric requirement had no inhibitory effect in gastric fistula dogs if time was allowed for gastric emptying. Beyond narrow limits, increasing caloric deficit was not effective in stimulating sham feeding; and the inhibition of subsequent food intake induced by sham feeding is of short duration.

If for purposes of analysis, we designate as 'oral' factors all the effects on the head receptors involved in the act of ingestion, and as 'gastric' factors the effects of gastric distention, the operation of these two sets of factors in regulating food intake during any one meal may be isolated and schematized. Thus, *a*) 'oral' and 'gastric' factors

are both involved in the regulation of food ingestion; *b*) 'oral' factors alone may produce cessation of eating, as in the dogs in which the continuity of the esophagus is interrupted, but a much greater stimulus is required than when the oral and gastric factors cooperate; and *c*) the 'gastric' factor is operative only in close association with the 'oral' factors. This analysis does not take into consideration the presence of other factors which undoubtedly exist. For example, the relationship between caloric deficit and food intake which manifests itself in long-term observations is not revealed by these studies.

So far as the results of these experiments reveal the mechanisms involved, the gastric factor appears to be purely mechanical, depending upon distention, and independent of absorption of nutrient materials.

The results of the sham-feeding observations are to be compared with those of sham drinking in dogs. Bellows (4) has reported that the amount of water sham-drunk is proportional to the size of the water deficit of the animal and that sham drinking is completely inhibited by intragastric instillation of the calculated water deficit after a latent period of about 10 minutes. In the present studies, intragastric feeding had no effect on subsequent sham eating under the standardized conditions of this experiment, and the amount sham-fed was apparently unrelated to size of the food deficit. These would appear to be important and fundamental differences between food-taking and water-taking behavior.

SUMMARY AND CONCLUSIONS

In *intact* dogs, the ingestion of a part of a meal 20 minutes before the *ad lib.* offering of the same food decreases the oral intake by an approximately equivalent amount. In *gastrostomized* dogs, the introduction of food into the stomach just before offering the regular oral meal decreases oral intake by an approximately equivalent amount. Inert material (Karaya gum) is just as effective as food in producing inhibition, indicating that the effect is a mechanical one due to distention. If the intragastric feeding is given several hours before oral feeding, no decrease in oral intake occurs. In *esophagostomized* dogs, sham feeding greatly exceeds food deficit. The placing of food into the stomach a few minutes or a few hours before does not inhibit sham feeding. The placing of food into the stomach *during* sham feeding does inhibit to some extent. Apparently, the factor of gastric distention can operate to reduce food intake only when it occurs simultaneously with oral ingestion. It is concluded that both oral and gastric factors operate and cooperate to regulate food intake during a meal.

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RESPONSE OF THE NORMAL DOG TO DIETARY SODIUM CHLORIDE

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IN THE dog, isotonic sodium chloride solution administered intravenously is rapidly excreted by renal mechanisms which involve an immediate and considerable rise in glomerular filtration rate (C_F) and effective renal plasma flow (RPF) (1). Increased ingestion of protein (2, 3) also induces a sustained increase in C_F and RPF and it is not clear to what extent this may involve increased salt intake. The present studies are therefore designed to examine the renal effects of changes in dietary salt alone.

METHODS

Five healthy female mongrel dogs weighing 14 to 17 kg. were placed on a diet of fixed composition and amount.² Water was supplied *ad libitum*. Exercise, time of feeding and other conditions of the experiment were kept constant as far as possible. After 2 to 4 weeks of control observations, salt intake was progressively increased to 9, 12, 18 and 27 gm. per day in 4 dogs, and to 40 and 60 gm. in 2 dogs. Each level of salt intake was maintained for at least one week. The dogs took diets containing 5 to 27 gm. of salt per day voluntarily, but higher intakes of salt required tube feeding and were achieved on only 3 dogs. In order to avoid vomiting, tube-fed diets were given in divided doses over a period of several hours during which time water intake was restricted. This water restriction coupled with salt administration resulted in a transient period of unavoidable hypertonicity of the body fluids, as judged by the plasma sodium and chloride concentrations.

During the control and experimental periods renal clearances and electrolyte excretion were measured at 2- to 4-day intervals. All reported observations represent the average of 3 to 4, 15-minute clearance periods taken 20 to 24 hours after the last meal. The filtration rate was measured by the exogenous creatinine clearance (4) and the effective renal plasma flow by p-aminohippurate (5). Sodium and potassium were determined with a Perkin-Elmer internal standard flame photometer model 52A. Chlorides were determined by Van Slyke's and Hiller's modification of the Sendroy iodometric titration (6).

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² One pound of Big Red Dog Chow per dog per day, containing 21 % protein, 1.14 % NaCl, and 0.81 % KCL. Originally 1½ pounds of food were given, but because 2 of the dogs showed weight gain the quantity of food was reduced. At this level, body weight remained constant. A sustained decrease in C_F and RPF resulted which was greater in magnitude than any increase observed post-absorptively during the subsequent experimental periods.

RESULTS

At intakes of 5 to 60 gm. of sodium chloride daily, the post-absorptive values of C_F , RPF and sodium excretion had invariably returned to the pre-existing level by the third day after an increase in salt intake. In some instances values obtained 24 hours after changing the salt intake were slightly greater than pre-existing levels, one dog consistently showing this type of response (fig. 1). The 24-hour increase in C_F was in no case greater than 25 per cent of the control value, and averaged 10 per cent. The magnitude of the increment in renal function bore no relationship to the size of the salt load. In 2 dogs daily intakes of 40 gm. of salt were achieved by intubation and in one of these it was later possible to maintain a level of 60 gm/day. Despite

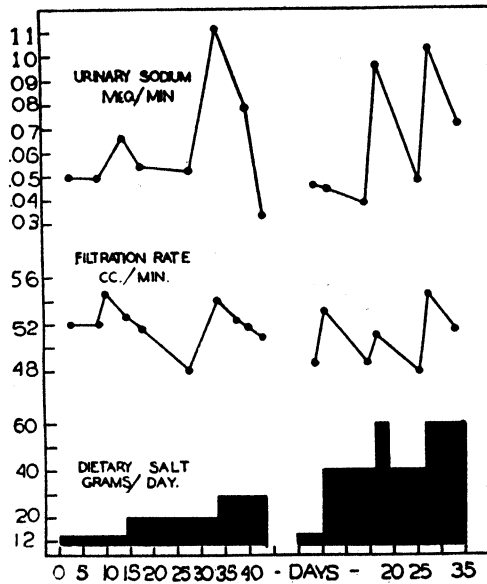


Fig. 1. RENAL FUNCTION 20 to 24 hours after feeding increasing quantities of salt. Each point is the average of 3 clearance periods.

this enormous intake no further changes in renal function or salt excretion were observed.

It is particularly noteworthy that throughout all our experiments no dog showed any evidence of edema formation or any significant increase in weight, and plasma sodium and chloride concentrations showed no more than small random variations.

In order to clarify the immediate pattern of salt excretion, C_F , RPF, and sodium excretion were followed in one dog at frequent intervals for 24 hours after the administration of 60 gm. of salt mixed with food. The dog had previously been on the control diet containing only 5 gm. of salt per day. The 24-hour study was repeated after the dog had been maintained on the 60-gm. diet for a week. On both occasions after salt administration there was a rapid and marked rise in C_F , RPF and sodium excretion (fig. 2), all functions returning to control levels between 12 and 24 hours.

Body weight was unchanged at the end of a week despite an increase in salt intake from 5 to 60 gm. of sodium chloride per day.

DISCUSSION

The absence of weight gain together with the constancy of plasma sodium and chloride concentrations show that no significant quantity of salt is retained by dogs when on a very high salt intake. Small increases in renal function are occasionally seen on the first day after the salt intake has been increased, but these persistent in-

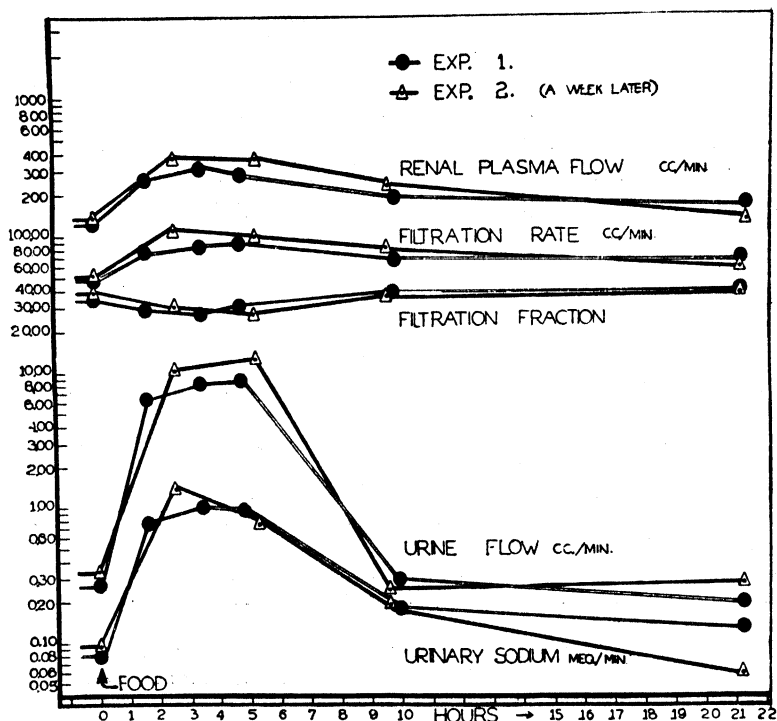


Fig. 2. IMMEDIATE CHANGES in renal function and salt excretion following a 60-gm. salt meal. Circles represent the responses after the first salt meal, triangles the responses after the diet had been maintained for one week. Each point is the average of 3 clearance periods.

crements invariably disappear by the third day after increase in salt load. If the 24-hour increments are significant, they perhaps indicate a gradual adjustment to high salt load which may have its explanation in changes in intestinal absorption, hormonal control of salt excretion or renal response. The rapid excretion of salt in the dog, even when administered in doses of 4 gm/kg/day, which corresponds to 280 gm/day for a 70-kg. man, is in marked contrast to the slower excretion in normal man (7-10), where relatively small salt loads (less than 0.5 gm/kg.) rapidly lead to weight gain and edema formation. Because of this difference between the species, it is hazardous to attempt to explain salt excretion in man solely by the physiological responses observed in the dog.

SUMMARY AND CONCLUSIONS

The oral administration to the dog of large quantities of salt (up to 4 gm/kg.) in association with the diet induces rapid and marked increases (up to 100%) in glomerular filtration and effective renal plasma flow, and the rapid excretion of salt (at rates in excess of 1 mEq/min.). Renal function may be slightly increased 24 hours later, but it invariably returns to normal by the third day of a continuing high salt diet. The renal excretion of salt by the dog is so efficient that intakes up to 4 gm/kg/day may be maintained for considerable periods (6 days) without increase in body weight or elevation of plasma sodium or chloride concentration, showing that there is no significant salt retention. This is in sharp contrast to man, where a moderate increase in salt intake (less than 0.5 gm/kg.) leads to significant retention of salt and water. Because of this difference between the two species it is hazardous to attempt to explain salt excretion in man solely by the physiological responses observed in the dog. The sustained elevation in renal function in the dog associated with a high protein diet is probably unrelated to any concurrent elevation in salt intake.

The authors wish to thank Dr. L. G. Wesson, Jr., and Dr. W. P. Anslow, Jr. for their valuable help and criticism.

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EXPERIMENTAL CARDIAC HYPERTROPHY: RATE OF DEVELOPMENT AND EFFECT OF ADRENALECTOMY¹

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THE rate of increase in the weight of the heart as a result of increased functional demand has not been adequately studied. The subject is treated in a cursory manner in the literature and textbooks of physiology and the time element, in general, ignored. The purpose of this paper is to present a statistical analysis of heart weights in young male albino rats subjected to an operation on the kidneys, originated by Grollman (1) on the basis of Page's earlier work (2), which increased cardiac work by inducing hypertension. It will be shown that there is a rapid increase in heart weight following the operation, demonstrable in as little as two days. It will also be shown that adrenalectomy interferes with the development of the increase in heart weight after the Grollman operation.

Since the estimation of slight changes in heart weight requires that this weight be related to some other body measurement, a preliminary discussion of this problem is necessary. As early as 1919 Klatt (3), in a mathematical analysis of the heart weights and body weights of 433 men and 368 women, showed that the relationship was of the form, heart weight = (constant) (body weight)^k. In his equations the value of the exponent k was 0.8181 for men and 0.7247 for women. Huxley (4) demonstrated the wide applicability of equations of the form $y = bx^k$, where y is the magnitude of the differentially growing organ and x the body weight, to problems of differential growth. He stated that the constant b had no biological significance and denoted only the fraction of x which y equalled when x was taken as unity. The value of the exponent k gives the ratio of rates of growth per unit weight of the organs concerned (or body as a whole). Changes in heart weight have been investigated by determining changes in *a*) the ratio of heart weight to body weight (5); *b*) the ratio of heart weight to surface area (6); and *c*) by the use of the exponential relationship discussed above (7). The objection to the first method is that if there are changes in the body weights of the animals during the course of the experiment the value of the ratio will change, independently of any effect due to the experimental procedure. This is because the value of the exponent k , for all species studied (3), is less than unity. Thus if the animals were to increase in weight the consequence of using this ratio might be to mask a small, but significant, increase in relative heart weight. To some extent this difficulty may be overcome by the use of controls, provided that the body weight changes are in the same direction and of the same magnitude. Also the importance of this factor would be at a minimum in short-term experiments. There is less objection to the use of the heart weight to body surface ratio, since the ratio of body surface to body weight decreases with increasing weight. In the study of the effect of operative procedures on the kidneys it is advantageous to use young, growing rats because of their relative freedom from spontaneous disease, particularly of the kidneys. The applicability of equations on exponential equation of the type under discussion to the colony of rats maintained at Stanford has been shown by Walter and Addis (7). The predicted heart weights used in the present study are taken from tables relating heart weight to body weight obtained by solving revised equations calculated from an enlargement of the original series (8).

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¹ This investigation was supported by a grant from the American Foundation for High Blood Pressure.

METHODS

The rats used were young male albinos of the Slonaker strain from the Stanford colony. They were selected in groups of 10, using litter mates when possible. Their ages ranged from 60 to 70 days and the initial body weights from 130 to 160 grams. The stock diet contained 18 per cent protein and was given *ad libitum*. The adrenalectomized animals were given 0.9 per cent saline *ad libitum* in addition. The rats were

TABLE 1. SUMMARY OF RESULTS: STANDARD ERRORS OF MEAN PERCENTAGE CHANGES IN HEART WEIGHTS FROM PREDICTED VALUES BASED ON KILL BODY WEIGHTS

OPERATION	NO. OF POST- OPERATIVE DAYS TO KILL DAY	NO. OF RATS	MEAN % CHANGE IN HEART WT. FROM PREDICTED VALUE	S. E. OF MEAN $\sqrt{\frac{s_D^2}{N-1}}$
Bilateral exposure and handling of kidneys	2	10	+0.4	1.5
	7	10	+2.5	1.6
	20	10	-1.2	1.4
Unilateral nephrectomy	2	10	+1.6	0.5
	7	10	+1.6	1.0
	20	10	-2.0	1.0
	40	10	+1.9	1.5
Unilateral nephrectomy + figure of 8 silk ligature around remaining kidney	2	10	+4.2	0.6
	5	8	+10.8	1.0
	10	8	+15.4	1.0
	20	8	+18.9	0.8
	40	10	+30.2	1.1
	120	8	+31.1	1.2
Bilateral adrenalectomy	10	10	+3.1	2.4
	20	21	+5.7	1.8
Bilateral adrenalectomy + unilateral nephrec- tomy	10	9	+6.9	3.0
	20	10	-0.7	3.0
Bilateral adrenalectomy, unilateral nephrectomy and figure of 8 ligature around remaining kid- ney	10	22	+13.1	3.0
	20	18	+7.2	2.0

caged in groups of 6 and kept in a ventilated room where the regulated temperature remained between 18 and 20° C.

OPERATIVE TECHNIQUE

Non-adrenalectomized Series. Aside from simple cleansing of the instruments and the use of alcohol on the operative site, sterile technique was not employed. No wound infections developed, however. The Grollman operation for inducing hypertension (1), consisting of unilateral nephrectomy and the application of a silk-thread figure of eight ligature to the remaining kidney, was the procedure adopted for

increasing cardiac work. The silk ligature was tied in such a manner as to compress the kidney gently. The time required to operate on 10 rats, with two operators² working simultaneously, was about 40 minutes. After the operation the animals were placed on the stock diet. They were killed in groups of 10 at 2, 5, 10, 20, 40 and 120 days post-operatively. The technique of the kill was uniform, the animals first being weighed and then anesthetized with ether, after which the aorta was exposed and cut. The hearts were removed in a uniform manner, the chambers opened and the muscle gently blotted on filter paper and weighed on a torsion balance. Two control series, one consisting of sham-operated animals in which the kidneys were exposed and handled, the other of unilaterally nephrectomized rats, were prepared. None of the controls died but of the 60 rats subjected to the Grollman operation 8 died within 48 hours of the operation. Data on these are not included in the tables.

Adrenalectomized Series. The Grollman operation group and the two control groups were prepared as before but at the time of operation simultaneous bilateral adrenalectomy was performed in addition on all rats. Only two kill points, at 10 and 20 days, were studied.

RESULTS

These are given in table 1 and figures 1 and 2. The increase in heart weight in the experimental series of non-adrenalectomized animals is of rapid onset so that there is a significant increase in weight within 2 days. Half of the total increase is reached within 10 days. The control series do not vary significantly from the predicted values. The data on percentage changes in body weight show that both the experimental group and the unilaterally nephrectomized group lose weight after the operation. This is only a temporary effect, however, and the slopes of the weight lines in figure 2 become approximately the same. In the adrenalectomized rats subjected to the same procedures the standard deviation of the individual heart weights was of a higher value than in the non-adrenalectomized rats. Larger numbers of animals were therefore used in order to diminish the standard error of the mean. The results show that the Grollman procedure did not produce increased heart weights in adrenalectomized rats killed 20 days post-operatively, although in the 10-day group there is an increase of probable significance.

HISTOLOGICAL FINDINGS

These will not be discussed in detail since they are not pertinent to the purpose of this paper. Perinephritis due to the silk ligature was indicated only by a lymphocytic infiltration of the renal capsule. No fibrous 'hull' developed. The silk thread produced a deep groove in the renal surface but could be removed without difficulty. The immediately adjacent tissue showed varying degrees of atrophy, fibrosis and lymphocytic infiltration. Aside from these areas, which were rather sharply localized, there was remarkably little change. Glomerular fibrosis and necrotizing arteriolitic changes of the malignant hypertensive type did not occur. Fibrinoid arteritis, involving extra-renal vessels of 200 to 1000 microns in diameter, was found in 3 rats of

² The author wishes to thank Mr. William Lew for his assistance at the operations and for his skillful care of the rats throughout.

the 120-day nephrectomy figure of eight group. The 3 rats with fibrinoid arteritis had hearts enlarged +67.6 per cent, +34.0 per cent and +32.5 per cent and in the first of these the mesenteric vessels were grossly involved in a manner similar to that described by Loomis (9) in rats after experimental renal infarction and subsequent hypertension. No attempt was made to study quantitatively changes in the diameter of cardiac muscle fibers. The histological sections showed no evidence of intra- or extracellular edema of the myocardium.

DISCUSSION

It might be objected that the increased heart weights demonstrated here in the early post-operative period are a consequence of post-operative weight loss unequally distributed between heart weight and total body weight. If such a differential weight loss were of appreciable degree it is true that the relative heart weight

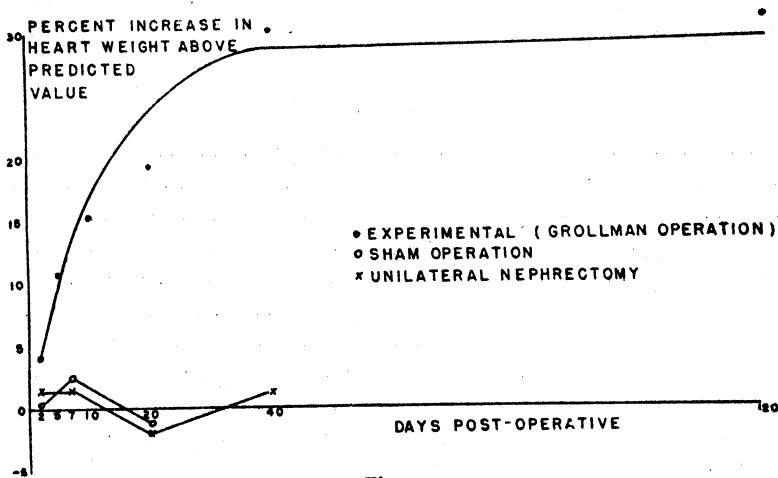


Fig. 1

would increase. But the relationship between heart weight and body weight is maintained with great tenacity even in the face of starvation. Walter and Addis (7) found that in 90 rats of the Stanford colony starved for 7 days, the actual heart weights had a mean value only 2 per cent in excess of the value predicted from the body weight, using an exponential formula. Addis, Poo and Lew (10) showed that after a 2-day fast the livers of their rats lost 20 per cent of their original protein content, while the kidney, heart and all other organs and tissues combined lost 4 per cent. The notion that the heart is 'spared' during starvation has been thoroughly disproved, although it continues to appear in most standard textbooks of physiology. Jackson (11) has reviewed the large amount of data, gathered chiefly during famines, which show the close dependence of heart weight on body weight in starvation.

Additional evidence for the validity of the early post-operative increase in heart weight in the animals subjected to the Grollman operation without adrenalectomy is the fact that the unilaterally nephrectomized animals showed no such increase, although they lost almost as much weight. (fig. 2.) Furthermore an examination of the

data in the tables will show that post-operative loss in body weight does not correlate with increase in relative heart weight.

Blood-pressure measurements were not made on any of these animals. What was under investigation was the rate of change of heart weight. It was assumed that the animals would develop hypertension and that hearts would subsequently increase in weight. In the animals studied here one may be sure that the increased heart weights reflect increased heart work, due to hypertension. The weights may, in fact, be more reliable than a few blood-pressure measurements. But is it true that if no weight increase is demonstrated we may suppose that no increased cardiac work demand and no hypertension were present? In other words, how sensitive is the heart weight with respect to heart work? On more or less *a priori* grounds we might expect the heart, under ideal circumstances, to be at all times just the 'right' size for the work it has to do. If such is the case then even a slight rise in diastolic pressure should result in an increase in heart weight. There is some evidence in favor of this view. Chanutin and Ferris (6) produced hypertension in rats by a technique involving subtotal nephrectomy. The average increase in the ratio of heart weight to surface area in 41 apparently well animals killed from 43 to 225 days post-operatively was 15.6 per cent, as compared with 51 control rats. The average increase in blood pressure was 44 mm. Hg. Their analysis of this data showed a high positive correlation between the heart weight over surface area ratio and the blood pressure. They concluded that cardiac hypertrophy was a constant and proportional accompaniment of hypertension produced in this way. Their data, however, are insufficient to answer the question of whether minor changes in diastolic pressures are reflected in heart weight. Nor can the rate of cardiac hypertrophy be determined from their figures since only one of the animals was killed in less than 70 days post-operatively. Hermann, Dechard and Erhard (5) using the ratio of heart weight to body weight, stated that in rats subjected to the unilateral application of a gauze-collodion bandage to one kidney followed in a few days by contralateral nephrectomy, the hypertrophy was largely completed in 40 days. Their series was unsuitable for the analysis of very early post-operative changes in heart weight.

If it is assumed that minor changes in elevation of the diastolic pressure will quickly give rise to an increase in heart weight then it becomes evident that unilateral nephrectomy in rats does not produce hypertension. Grollman (12), on the other hand, has stated that following unilateral nephrectomy the majority of rats show a slightly elevated level (average 8 mm.) when studied in groups large enough for this difference to be of statistical significance. It may be that his mean value is being pulled up by an occasional animal with disease of the remaining kidney leading to the development of hypertension. This seems to be the view adopted by Grollman (13) somewhat later. Grollman (14) also states that an immediate rise in blood pressure does not occur after the application of a figure of eight ligature and that it is only after some weeks that the effect becomes evident. This is in striking disagreement with the data on heart weights presented in the present paper.

The effect of adrenalectomy in preventing the development of experimental renal hypertension is generally accepted as proven (15) although there has been some dissent. Braun-Menendez *et al.* (16) have discussed the evidence for the view that

the effect of adrenalectomy on the development of hypertension is due to a reduction in the amount of renin substrate in the blood. It has been shown that adrenalectomized rats are less sensitive to renin than normal rats (17), and that in dogs in adrenal insufficiency there is less of a pressor response to renin than in normal dogs. This reduction in sensitivity to renin did not appear until several days after adrenalectomy (18). The data on heart weights in adrenalectomized rats presented above are compatible with the hypothesis that an early hypertension occurred after the Grollman operation, leading to a small, though probably significant, increase in heart weight at the end of 10 days, but that the hypertension was not maintained, hence the lack of significant difference in mean heart weights of the experimental and control series at the end of 20 days.

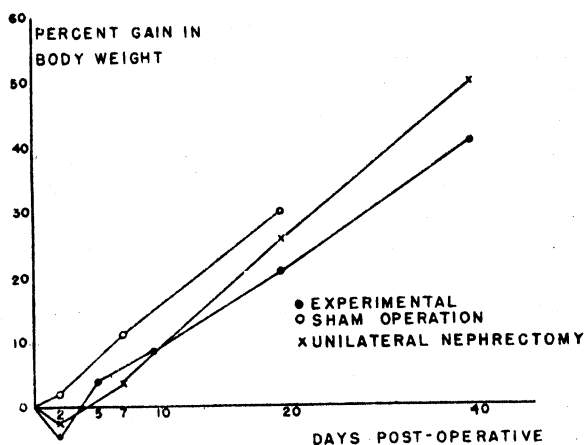


Fig. 2

SUMMARY

In albino rats subjected to the Grollman operation for inducing hypertension (unilateral nephrectomy and ligature around the remaining kidney) cardiac hypertrophy developed with great rapidity. In a series of animals killed in groups at intervals of 2 to 120 days post-operatively a significant increase in heart weight appeared as early as 2 days and one half of the ultimate increase was reached in 10 days. No significant progression of hypertrophy occurred after 40 days. None of the animals developed glomerular fibrosis or necrotizing arteriolitis. Three of 8 rats killed at 120 days had fibrinoid arteritis involving extra-renal vessels of 200 to 1000 microns in diameter. Control series of unilaterally nephrectomized and sham-operated rats were used. The heart weights in these did not vary from the predicted values. Comparably prepared animals subjected also to bilateral adrenalectomy showed significant cardiac hypertrophy at 10 days but at 20 days did not differ from the control groups. It is suggested that in the adrenalectomized animals the hypertensive load was not maintained.

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EFFECT OF HYPERTONIC SOLUTIONS ON METABOLISM AND EXCRETION OF ELECTROLYTES

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THAT urine flow increases after the administration of hypertonic solutions under conditions of water deprivation has long been known (1, 2). More recently, it has been demonstrated that certain solutes, such as glucose, sucrose, sorbitol, mannitol, and sometimes urea, accelerate the excretion of sodium and chloride as well as water (3-7). These effects on water and salt excretion have been ascribed almost entirely to the osmotic action within the renal tubules of that fraction of the administered solute which appears in the urine, without particular reference to changes in composition or volume of body fluid or to intracellular exchanges.

The present investigation describes some of the effects of the administration of hypertonic solutions on the volume and composition of the extracellular fluid, on cellular transfers and on the metabolism and excretion of water and electrolytes. The extent and character of the changes, and their possible interrelation, were studied by using solutes with different metabolic and osmotic properties.

METHODS AND CALCULATIONS

Glucose was determined, in whole blood, by the method of Benedict using zinc filtrates (8, 9), and in urine by the method of Somogyi (10). Mannitol was determined by the method of Smith, Finkelstein and Smith, as modified by Elkinton (11). Inorganic phosphorus was determined by the method of Fiske and Subbarow (12) as modified for the photoelectric colorimeter. Other methods used were identical with those previously reported from this laboratory (13, 14).

When glucose was given, serum glucose was calculated from the values for whole blood by correcting for the solids in red blood cells (15): Blood glucose mm/l. $\div 0.85$ = serum glucose mm/l. Urea concentrations were calculated from the total non-protein nitrogen of blood and urine by the use of standard correction factors for non-urea nitrogen. The rate of excretion on non-protein nitrogen in the urine and the concentration of non-protein nitrogen in the blood during the control period were multiplied by 0.15 and 0.40, respectively, to give values for non-urea nitrogen. These values were subtracted from subsequent excretion rates and concentrations in blood and expressed as urea. Values thus calculated for urea concentrations of random urines agreed within 5 per cent with direct measurements of urea by the gasometric method of Van Slyke (16). It was assumed that non-protein nitrogen other than urea was not altered by injections of urea.

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Balance data were calculated by the methods previously reported from this laboratory (17, 18) and by Darrow (19). Balance periods, designated in table 3, represent intervals between the withdrawal of specimens of blood. These periods, therefore, may include several collections of urine, which are listed individually in tables 1 and 2.

To estimate the effect of expansion or contraction of the extracellular volume alone on the concentration of serum potassium, the following expression was used:

$$D = K_{s_i} (EC_i - EC_f)$$

D = dilution (or contraction) effect, in milliequivalents. K_{s_i} = initial concentration of serum potassium in milliequivalents per liter. EC_i , EC_f = initial and final extracellular volume in liters, as estimated from changes in the chloride space. The expression gives the milliequivalents of potassium that would have to be introduced into, or removed from, the extracellular fluid in order to maintain a constant concentration of serum potassium, if neither urinary losses nor cellular transfers intervened. 'Loss' of potassium, as a result of dilution, has a positive sign; 'gain' of potassium, as a result of contraction of the extracellular volume, has a negative sign. The algebraic sum of urinary losses and cellular transfers of potassium plus the number of milliequivalents 'gained' or 'lost' as a result of contraction or expansion of extracellular fluid represents the total number of milliequivalents of potassium which must be removed from, or added to, the extracellular fluid to produce a given change in the concentration of serum potassium.

EXPERIMENTAL PROCEDURE

The subjects of the experiments were 4 healthy young adult males and one woman, *P. B.*, who had an islet-cell adenoma of the pancreas, but was otherwise in good physical condition and free from evidences of renal disease. Solutions of glucose, mannitol, urea and urea plus sodium chloride were injected intravenously or ingested while the subjects were thirsting (dehydration) and while they were taking water (hydration). The composition and route of administration of these solutions are recorded in tables 1 and 2.

In the dehydration experiments the subject, after a 15-hour period without food or fluids, remained in a semi-reclining position on a couch. The first urine, voided after an hour, was discarded. Several subsequent control urines were usually obtained to ascertain that the excretion of water and electrolytes had become stabilized. Data for only the last control period are recorded, except that in the case of *P. B.* a single prolonged control urine was used. At the end of the control period a blood specimen was drawn, after which the injection or ingestion of hypertonic solution was begun. Additional specimens were obtained at the time of voidings on two or three occasions after the cessation of the infusion.

In the hydration experiments the same general procedure was followed; but 400 cc. of tap water per hour were taken for 5 hours before and throughout the experiment. In two additional experiments the effect on electrolyte excretion of drinking 1300 cc. of tap water per hour was studied for comparison.

Venous thromboses occurred in every instance after injection of urea and in

TABLE 1. EFFECT OF INFUSIONS OF GLUCOSE, MANNITOL, UREA, OR UREA AND NA CL IN 9 EXPERIMENTS: ANALYSES OF SERUM, BLOOD, AND URINE AND CHANGES IN URINE FLOW

EXPER. SUBJECT, WT.	HYDRATION	SOLUTION INJECTED ¹	PERIOD ²	DURATION ³ min.	SERUM					URINE									
					Cl	Na	K	P	Gluc. ⁴ Mann. or Urea	Osmo- lar ⁴ Conc.	Flow	Cl	Na		K	P	Urea, Gluc. ⁴ or Mann.	Osmo- lar ⁴ Conc.	
					mEq/l.	mEq/l.	mEq/l.	mg/l.	mM/l.	mM/l.	cc/lw.	mEq/ lw.	mEq/l.	mEq/l.	mEq/l.	mEq/l.	mEq/ lw.	mM/ lw.	mM/l.
1, P.B., 71.5 kg.	Hydro- penia	750 cc. of 25% gluc. in 39 min.	Control	463	101.7	138.5	4.4	37.8	4	145	35	3.3	2.3	64.7	2.1	59.8	17.6	482	
			1	44	91.0	125.0	4.3	36.9	60	159	580	20.8	17.7	30.5	4.4	7.6	46.6	135	347
			2	30							830	25.0	22.2	26.8	3.2	3.9	39.4	200	324
			3	46	101.1	135.7	4.0	23.4	23	151	391	13.3	11.8	30.2	2.7	6.8	31.1	113	410
2, R.T., 60.5 kg.	Hydro- penia	1000 cc. of 25% gluc. in 88 min.	Control	60	99.6	138.4	4.2				47	10.4	7.6	101.0	4.7	99.8		895	
			1	91							277	19.4	15.8	57.0	5.8	20.8		57	433
			2	30	99.0	136.4	4.4				636	34.3	30.2	47.5	4.4	6.9		154	384
			3	104	101.6	141.5	3.9				81	10.0	7.8	96.5	2.5	30.6		15	626
3, D.W.S., 63.2, kg.	Hydro- penia	1000 cc. of 25% gluc. in 74 min.	Control	75	103.0	139.1	4.1		6	146	38	8.9	8.8	232.3	2.4	64.2		975	
			1	55							546	31.4	30.2	55.4	5.4	9.9		131	421
			2	28	98.7	136.8	4.1		36	159	996	45.0	39.6	39.7	4.5	4.5		194	305
			3	37							321	21.4	19.7	61.5	3.8	11.8		80	451
			4	68	104.0	146.2	3.8		3	152	39	6.5	5.6	143.4	3.4	87.7		2	814
4, F.D.L., 65.0, kg.	Water diure- sis	100 cc. of 25% gluc., then 500 cc. of 25% gluc. in 46 min.	Control	46	93.2	131.0	3.8		7	138	588	6.0	5.5	9.2	2.9	4.9		48	
			1	27							558	11.0	10.9	19.4	2.0	3.5		45	142
			2	28	85.8	116.3	3.9		51	146	1105	26.4	27.3	24.7	2.0	1.8		131	182
			3	25							1462	30.7	30.6	21.2	1.9	1.3		152	158
			4	40							840	24.0	23.3	27.9	1.2	1.4		93	183
			5	25	93.8	131.9	3.0		17	143	840	31.6	31.1	37.0	1.5	1.8		101	219

5, D.W.S.	Hydro- penia	400 cc. of 25% mannitol in 15 min.	Control 1 2 3 4	30 25 38 29 81	106.7 143.7 96.9 129.5 102.7 135.4 103.5 137.0	4.1 4.0 4.5 4.2	35.6 32.1	148 150 32	78 571 463 372 242	17.6 44.1 32.8 30.9 23.8	16.1 206.4 39.2 68.8 29.4 63.6 26.9 72.4 21.1 87.3	4.9 6.9 4.0 4.9 6.1	62.5 12.1 8.6 13.1 25.0	48.6 4.6 1.4 1.1 1.9	736 431 373 410 516	
6, R.T.	Water diure- sis	400 cc. of 25% mannitol in 40 min.	Control 1 2 3 4 5 6	34 31 21 11 44 64	103.0 140.0 96.4 129.3 96.4 129.8 96.9 131.5	3.8 3.9 3.8 3.9	32.7 29.0 33.3 34.1	144 148 30 14 8	676 862 728 606 495 473 469	16.5 33.8 42.9 36.0 28.8 27.3 23.6	15.9 31.4 39.7 35.0 28.0 27.9 24.3	23.5 36.4 54.5 57.8 56.5 59.0 51.8	3.7 4.5 3.0 2.7 2.2 2.6 3.1	5.5 4.5 4.1 4.4 4.5 5.5 6.7	17.2 6.9 2.2 1.8 1.5 1.4 8.2	89 228 371 326 368 344 275
7, D.W.S. ⁷	Hydro- penia	500 cc. of 10% urea in 37 min.	Control 1 2 3 4	54 34 19 39 36	100.7 145.3 98.8 140.3 99.6 137.9	4.5 4.3 4.1		9 154 156 152	43 113 278 134 112	9.9 13.6 8.9 4.4 4.1	9.0 208.3 12.4 109.8 5.5 19.8 1.5 11.5 1.6 14.3	5.6 6.2 5.5 3.9 3.1	128.8 54.7 19.6 29.0 27.5		12 43 91 406 51 461 56 584	
8, R.T. ⁷	Water diure- sis	500 cc. of 10% urea in 44 min.	Control 1 2 3 4	21 34 35 26 24	99.5 138.4 97.2 135.8 97.6 134.6	3.8 4.1 3.9		6 27 21	549 527 473 288 370	8.5 13.3 15.0 11.7 8.7	11.3 20.5 15.2 28.8 15.4 32.5 11.5 40.1 8.6 23.2	3.4 2.7 2.4 2.1 2.7	6.2 5.2 5.0 7.2 7.4		16 63 187 323 426 83 286	
9, E.A.	Hydro- penia	1325 cc. of 5% urea and 2% NaCl in 174 min.	Control 1 2 3 4	43 78 99 45 28	101.1 137.2 111.0 146.2 111.7 149.2	4.6 4.6 4.5		7 26 23	46 103 188 236 219	12.5 19.2 28.0 37.2 35.0	10.9 237.6 15.3 149.0 22.7 120.8 29.8 126.5 29.0 132.2	4.7 5.0 6.7 7.9 5.6	102.5 48.6 35.7 33.6 25.5		15 49 83 99 94 745	

¹ Injection started immediately after end of control period. ² Blood specimens drawn at ends of corresponding urine-collection periods. ³ Con-
centration of mannitol in serum directly determined. Concentrations of glucose and urea in serum calculated from blood glucose and blood NPN, re-
spectively. ⁴ Represents the sum of serum concentrations of sodium and potassium and $\frac{1}{2}$ serum concentration of substance injected. ⁵ Excretion
rate of urea is given only in experiments when urea was injected. ⁶ Represents sum of urine concentrations of substance injected and urea, plus twice
the sum of urine concentrations of sodium and potassium. ⁷ Urea was administered in a solution of 5 per cent glucose.

one instance after glucose (*experiment 4*, table 1). Frank hypoglycemic reactions occurred at the end of *experiments 2* and *3*, table 1.

RESULTS

Analytical data from the experiments in which solutions were injected and ingested are presented in tables 1 and 2, respectively; derived data in table 3.

TABLE 2.¹ EFFECT OF INGESTION OF WATER, OR UREA AND SODIUM CHLORIDE: ANALYSES OF SERUM, BLOOD, AND URINE AND CHANGES IN URINE FLOW

EXPER. ² SUBJECT	SOLUTION TAKEN	PERIOD	DURATION	SERUM			URINE										UREA, GLUC. OR MANN.	OSMOLAR CONC.
				Cl	Na	K	Flow	Cl	Na		K		UREA, GLUC. OR MANN.		OSMOLAR CONC.			
			min.	mEq/l.	mEq/l.	mEq/ l.	cc/hr.	mEq/ hr.	mEq/ hr.	mEq/ hr.	mEq/l.	mEq/ hr.	mEq/ l.	mEq/ hr.	mEq/ l.	mM/ hr.	mM/ l.	
10, D.W.S.	Water	Control	85	102.5	139.3	4.6	35	6.8	6.2	176.0	2.2	63.5					955	
	p.o.	1	89				297	11.3	11.0	37.1	5.4	18.1					183	
	1250	2	61		136.9	4.3	798	7.5	6.8	8.5	6.6	8.3					59	
	cc/hr.	5	18				709	5.6	5.4	7.6	4.5	6.3					56	
		6	24	100.2	138.4	4.2	631	4.5	3.8	6.0	4.0	6.4					54	
11, R.T.	Water,	Control	68	102.4	138.8	3.8	40	9.2	7.2	179.0	2.8	71.1					924	
	p.o.	1	151	103.3	139.5	3.8	447	10.3	8.3	18.6	4.7	10.6					98	
	1330	2	22				900	8.8	8.9	9.9	4.2	4.7					47	
	cc/hr.	4	19				973	7.6	9.8	10.1	3.2	3.3					45	
		5	21	97.3	139.5	3.5	948	7.3	9.5	10.0	2.4	2.5					42	
12, D.W.S.	5% urea,	Control	37	103.0	140.7	4.8	24	4.9	4.3	177.2	1.4	57.1	13				997	
	2%	1	60				60	12.9	11.6	192.7	2.9	47.9	30				978	
	NaCl,	2	35				110	20.5	17.3	157.1	5.0	45.3	55				909	
	p.o. at	3	35				161	28.2	23.7	147.2	6.1	37.7	71				813	
	242	4	57	106.3	143.4	4.4	163	30.8	26.4	161.8	6.0	36.8	67				808	
13, R.T.	5% urea,	Control	44		141.8	4.3	48	8.6	9.3	194.5	5.1	107.0	15				915	
	1	1	120				79	20.1	12.4	157.4	6.7	85.2	29				852	
	2%	2	138				73	18.3	13.0	178.0	3.0	40.6	41				999	
	NaCl,	3	49		146.8	3.9	67	18.0	9.6	142.9	2.9	22.4	39				960	
	p.o. at																	
203																		
cc/hr.																		

¹ See footnotes of table 1.

² Diarrhea occurred during *experiments 12* and *13*.

Alterations in Body Fluids. The concentration of serum sodium fell 11 to 15 mEq., and extracellular volume expanded 0.5 to 1.5 liters, after the cessation of the infusion, in 2 of 4 glucose experiments and in both mannitol experiments (tables 1 and 3). In *experiments 2* and *3*, where glucose was injected comparatively slowly, there was no expansion of extracellular volume and the serum sodium fell only

slightly. Injections of urea produced a slight but definite diminution in the concentration of serum sodium without expanding extracellular volume. Despite the fall

TABLE 3. BALANCES OF ELECTROLYTES AND GLUCOSE AND CHANGES IN EXTRACELLULAR VOLUME IN 9 INTRAVENOUS EXPERIMENTS

EXPER., SUBJECT, WT.	SOLUTION INJECTED	BAL- ANCE PE- RIOD ¹	EXTERNAL BALANCE ²					CHANGE IN EXTRA- CELLU- LAR VOLUME ³	EXTRACELLULAR BALANCE				INTRACELLULAR BALANCE			
			Cl	Na	K	P	Glucose		Na	K	P	Glucose ⁴	Na	K	P	Glucose ⁴
			mEq.	mEq.	mEq.	mg.	gm.		mEq.	mEq.	mg.	gm.	mEq.	mEq.	mg.	gm.
1, P. B., 71.5 kg.	750 cc. of 25% glucose	A	-18	-17	-3	-34	+170	+1.5	-9	+5	+42	+118	-8	-8	-76	+52
		B	-25	-25	-4	-44	-34	-1.8	-81	-12	-255	-84	+56	+8	+211	+50
2, R. T., 60.5 kg.	1000 cc. of 25% glucose	A	-48	-40	-11			-0.4	-79	0			+39	-11		
		B	-18	-15	-4			-0.4	-2	-7			-13	+3		
3, D. W. S., 63.2 kg.	1000 cc. of 25% glucose	A	-51	-48	-7		+212	+0.1	-15	0		+49	-33	-7		+163
		B	-22	-20	-6		-9	-0.8	0	-7		-53	-20	+1		+44 ml
4, F. D. L., 65.0 kg.	100 cc. of 50% glucose; 500 cc. of 25% glucose	A	-18	-19	-2		+160	+0.9	-90	+5		+78	+71	-7		+82
		B	-43	-43	-2		-30	-1.6	+5	-17		-63	-48	+15		+33
5, D. W. S.	400 cc. of 25% mannitol	A	-20	-19	-3	-2		+1.1	-37	+3	-8		+18	-6		+6
		B	-39	-35	-5	-2		-1.1	-70	+2	-6		+35	-7		+4
		C	-35	-32	-8	-3		-0.4	-34	-6	+5		+2	-2		-8
6, R. T.	400 cc. of 25% mannitol	A	-35	-33	-3	-5		+0.5	-65	+3	-29		+32	-6		+24
		B	-42	-42	-3	-2		-0.4	-50	-3	+39		+8	0		-41
		C	-27	-29	-3	-9		-0.3	-19	0	-1		-10	-3		-8
7, D. W. S.	500 cc. of 10% urea	A	-11	-10	-5			+0.1	-49	-2			+39	-3		
		B	-6	-3	-4			-0.1	-48	-3			+45	-1		
8, R. T.	500 cc. of 10% urea	A	-17	-19	-3			+0.1	-20	+4			+1	-7		
		B	-10	-10	-2			-0.1	-29	-3			+19	+1		
9, E. A., 77.3 kg.	1325 cc. of 5% urea and 2% NaCl	A	+381	+396	-18			+1.6	+382	+8			+14	-26		
		B	-45	-37	-9			-0.4	-10	-4			-27	-5		

¹ Balance data are expressed per individual balance period rather than cumulatively. Duration of balance period is the interval between withdrawals of blood specimens, as shown in table 1.

² Balances of chloride and sodium are corrected for small quantities lost in serum drawn for analysis.

³ Calculated from the changes in the chloride balance after assuming initial extracellular volume of 20% of body weight.

⁴ Average glucose concentration of extracellular fluid assumed to be 20% less than that of whole blood (20).

⁵ Positive intracellular balances of glucose represent storage or utilization.

in the concentration of sodium salts, the osmolar concentration of the serum rose in all experiments (table 1).

Excretion of Sodium. Infusions of glucose or mannitol caused striking increases in the excretion of sodium during dehydration and hydration. Urea, although excreted at rates and concentrations comparable to those of glucose or mannitol, had little effect on the excretion of sodium. A minimal transitory increase in the excretion of sodium was followed in *experiment 7* by a fall to values well below those of

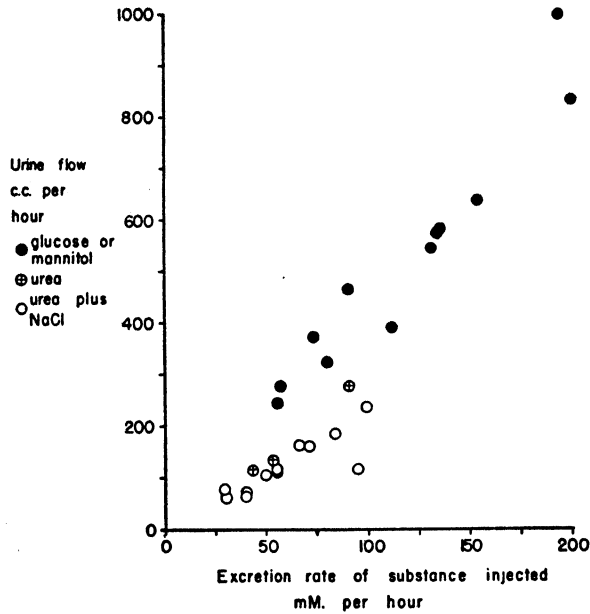


Fig. 1. RELATION OF EXCRETION rate of administered solute to urine flow during hydropenia. When sodium chloride was given with urea the values are plotted in terms of urea alone as open circles.

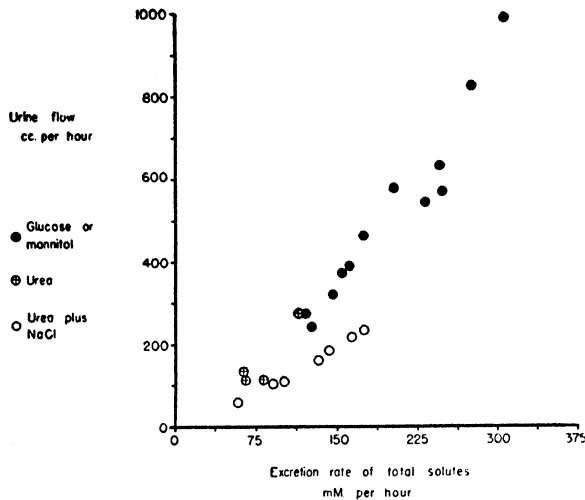


Fig. 2. RELATION OF EXCRETION rate of total urinary solutes to urine flow during hydropenia.

the control period (table 1). There was a similar trend in *experiment 8*. A diuresis produced by the ingestion of water alone had no significant effect on sodium excretion, save for a slight initial increase (table 2).

Excretion of Water. Urine flow varied directly with the excretion rate of injected solute (fig. 1) or total solutes (fig. 2) during dehydration. Urea differed from glucose

or mannitol in commanding less water for its excretion (fig. 1). This was not the result of a decreased excretion of total solutes during urea diuresis, since the amount of water commanded by each milliosmol of urinary solute was less when urea was the principal urinary constituent than when the urine contained large amounts of glucose or mannitol (fig. 2). The one exceptional point in figure 2 is from *experiment 7* where urea was injected with water and may have produced a mild water diuresis, since Verney has shown that urea does not stimulate the cerebral osmoreceptors as other solutes do (21).

If consecutive periods of a given experiment during hydropenia are compared, the concentration of total solutes in the urine varies inversely with urine flow (tables

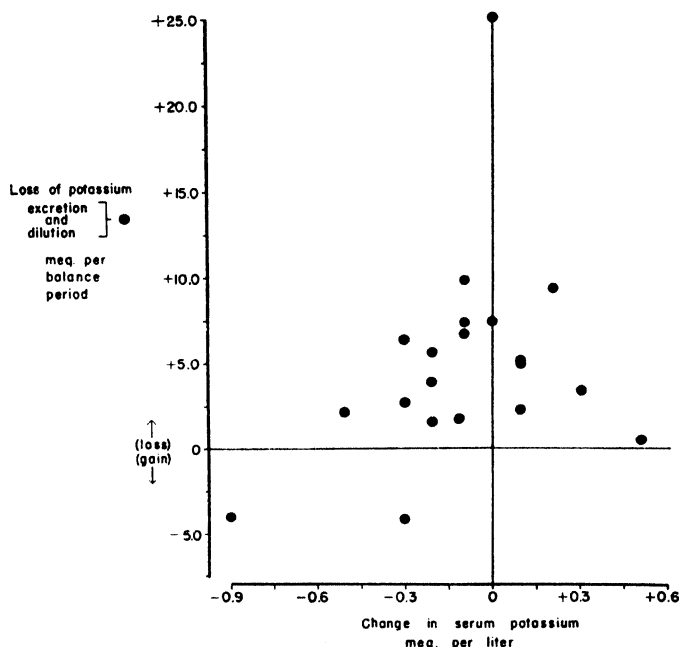


Fig. 3. RELATION OF EXCRETION plus dilution to changes in the concentration of serum potassium.

1 and 2). This relationship is less rigid if the comparison is made between periods from different experiments.

Transfers and Excretion of Potassium. The absence of correlation between the sum of urinary losses and dilution effect and changes in the serum concentration is evident from figure 3. Cellular transfers of potassium counteracted the diluting or concentration effect of expansion or contraction of extracellular volume. Apparently, the rate of release of potassium from cells is slowed or accelerated as extracellular volume contracts or expands, thereby preventing changes in the serum concentration (tables 1 and 3). The single exception to this principle occurred during balance period B of *experiment 5* (tables 1 and 3), when serum potassium rose by 0.5 mEq/l. as extracellular volume diminished by 1.1 liter. However, continued shrinkage of extracellular volume during balance period C was associated with a

fall in serum potassium which almost completely counterbalanced the preceding rise. The net effect then was a marked decrease in extracellular volume while serum potassium changed insignificantly.

When glucose was given the concentration of serum potassium varied in a manner which suggested that metabolic factors modified purely osmotic effects. As glucose utilization or storage was accelerated during the second balance period of the experiments the concentration of serum potassium usually fell (tables 1 and 3). This

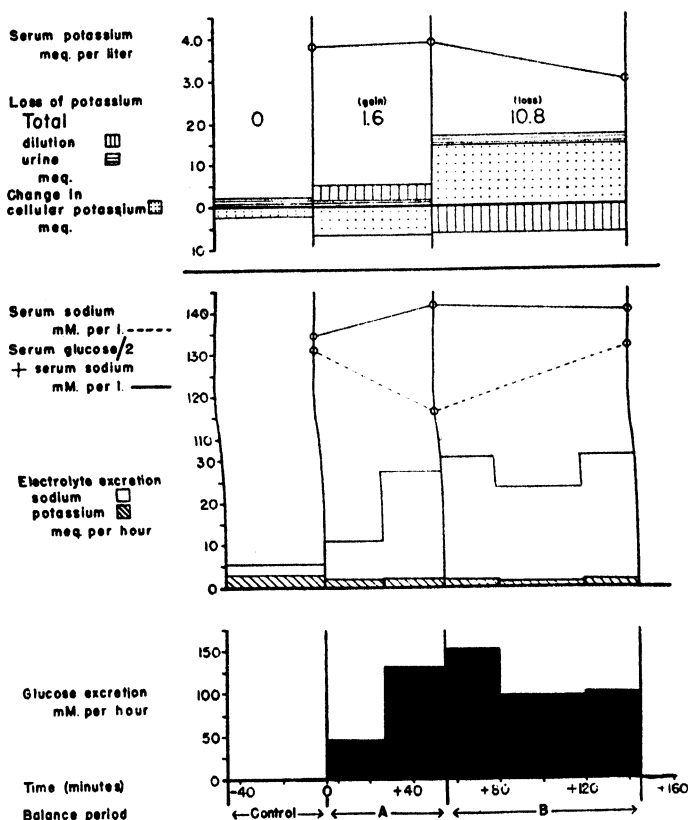


Fig. 4. EFFECT OF INJECTION of glucose on the metabolism and excretion of sodium and potassium (*experiment 4*).

probably reflects movements of potassium required for the metabolism of glucose, particularly in view of the parallelism between the exchanges of phosphorus and those of potassium (*experiment 1*, tables 1 and 2). *Experiment 4* is a striking demonstration of the influence of glucose on the metabolism of potassium (fig. 4). Serum potassium did not fall during period A despite a calculated dilution effect of 3 mEq. and a urinary loss of 2 mEq., because 7 mEq. of potassium left the intracellular phase. During period B, only 2 mEq. were excreted while extracellular volume contracted by 1.6 liters, thereby resulting in a net 'gain' of 4 mEq. from the standpoint of serum concentration. Nevertheless, the concentration of serum potassium fell by 25 per cent because of a transfer of 15 mEq. of potassium into cells. The net effects

of dilution and renal loss, therefore, failed to account for the changes in the concentration of serum potassium. The fall in the concentration of serum potassium was a reflection of a movement of potassium associated with the metabolism of glucose (table 3). The values listed as 'total' (fig. 4) represent the algebraic sum of all losses or gains with respect to the concentration of serum potassium and must account for the observed changes in concentration of serum potassium.

The excretion of potassium was remarkably stable during these experiments despite a marked augmentation in the excretion of water and sodium. If the hypertonic solution was injected during dehydration, the urinary losses of potassium were slightly accelerated. This has been noted during the diuresis produced by many other solutes and is probably due to the dehydrating effects of the hypertonic solution (17). If dehydration was ameliorated by the ingestion of water, urinary losses of potassium fell slightly. The mechanism of the slight augmentation in the excretion of potassium during ingestion of water alone is not clear since the subjects were in positive water balance (table 2). During water diuresis (*experiment 11*) and glucose diuresis (*experiments 1* and *4*), the ratio of the concentration of potassium in the urine to that in serum (U/P ratio) was less than one (22).

Excretion of Phosphorus. The injection of mannitol depressed the excretion of phosphorus, whereas glucose augmented it (table 1). The augmentation of the excretion of phosphorus when glucose was injected was also observed during similar experiments in edematous patients (23) and after the administration of glucose when it resulted in insignificant glycosuria (24).

DISCUSSION

The profound fall in the concentration of serum sodium in two of four glucose experiments and in both mannitol experiments, in contrast to the minor depression following the injection of urea, demonstrates the highly differentiated response to osmotic distortions of the internal environment. Urea is distributed throughout the total body fluid, and cannot alter the distribution of water between the cells and extracellular fluid, since it contributes equally to the osmotic pressure of both phases. Injections of urea, therefore, do not expand extracellular volume (table 3, *experiments 7* and *8*), and produce only a minor fall in the concentration of serum sodium.

In contrast to urea, injections of glucose or mannitol produce marked dislocations in the distribution of body fluids. In *experiments 1* and *4* (table 1), where glucose was injected very rapidly, the concentration of serum sodium had fallen about 15 mEq. at the end of the infusion. This deficit was too large to be ascribed to dilution by the injected fluid. Increased urinary losses of sodium and water could not account for the fall, since the quantity of sodium excreted was too small and its concentration was distinctly lower in the urine than in the serum. There was no movement of sodium into cells in *experiment 1* and the calculated transfer in *experiment 4* was much too small to cause the deficit. Moreover, neither cellular transfer of sodium nor urinary losses could explain the expansion of extracellular volume which occurred when the serum sodium was depressed. Therefore, the fall in the concentration of serum sodium and the concomitant expansion of extracellular volume must be due

to a withdrawal of water from cells in response to the increase of the osmotic pressure of the extracellular fluid caused by high concentrations of glucose. Such alterations in the distribution of water between cells and extracellular fluid would not occur if glucose freely traversed cellular membranes and came to a diffusion equilibrium. The failure to recover large increments of the injected glucose in the extracellular fluid or urine (table 3) indicates that glucose has been metabolized, the free glucose being confined largely to the extracellular fluid. This is consistent with Cori's demonstration by direct analysis of the extremely low concentration of free glucose in rabbit muscle (25).

When glucose was given at a comparatively slow rate in *experiments 2* and *3* (table 1), the serum sodium fell only slightly and the chloride space did not change appreciably. Since the osmotic pressure of the serum was considerably above the initial values, and no significant amount of water could be demonstrated to have left cells, it is reasonable to assume that increased utilization of glucose resulted in a rise in the concentration and quantity of osmotically active constituents of cells which thereby preserved a uniform osmotic pressure. This is supported by the fact that the movement of glucose into cells, when blood was drawn for analysis, was two to three times greater in *experiment 3* than in *experiments 1* and *4* (table 3). Moreover, during the last period of *experiment 1*, the serum sodium, which had been depressed from 138.5 to 125.0 mEq/l. when the concentration of serum glucose was high, returned almost to the initial values despite the persistence of 23 mm/l. of glucose in the serum. Here, too, apparently, increased utilization of glucose promoted an increase in the osmotic pressure of cells so as to maintain osmotic equilibrium without large shifts of water. These findings are in accord with the demonstration of previous workers (26) that alterations in the quantity of osmotically active constituents of cells contribute to the maintenance of a uniform osmotic pressure.

The behavior of mannitol, which seems to be confined essentially to the extracellular fluid (11), was similar to glucose in withdrawing water from cells, expanding extracellular volume, and diluting serum sodium. The serum sodium remained depressed, however, in the later periods of the experiments, when only 8 to 10 mm of mannitol remained in the serum (table 1, *experiments 5* and *6*). Similar amounts of glucose did not prevent the return of the serum sodium to its initial value in the final periods of *experiments 1* and *4*. This suggests that the increase in the osmotically active constituents of cells, which contributed to the maintenance of osmotic equilibrium when glucose was given, was not as significant a factor in maintaining osmotic equilibrium when a metabolically inert substance such as mannitol was administered.

Synchronous with the reduction in the concentration of serum sodium produced by glucose and mannitol, the excretion of salt increased sharply. If this were the result of the osmotic action of glucose or mannitol within the renal tubule, the excretion of comparable amounts of urea should produce a similar acceleration of sodium excretion. But urea had no significant effect. This discrepancy suggests that the dislocations in the internal environment produced by solutes whose distribution is essentially extracellular (such as glucose or mannitol) is a regulatory factor in the excretion of salt. In 1923, Gamble, Ross, and Tisdall (27) demonstrated that the electrolyte adjustments mediated by the kidney during starvation preserved

a constant concentration of fixed base (which consists almost entirely of sodium) in the serum. The remarkable stability of the concentration of serum sodium under a diversity of circumstances suggests that alteration of the serum sodium is a regulatory factor controlling sodium excretion. However, as Gamble subsequently indicated (28), the osmotic value of the extracellular fluid is usually determined almost completely by the concentration of sodium salts, so that it would be difficult to determine which factor is operative. This difficulty is circumvented in the present study by the marked dissociation between the concentration of sodium salts and the osmotic pressure of the serum as a result of high concentrations of the injected solute which contribute considerably to the total osmotic pressure. The excretion of sodium was usually inversely related to the concentration of serum sodium, whereas the serum osmotic pressure, since it was determined by the concentration of sodium salts and the injected solute, was directly related to changes in sodium excretion. From this it would appear that a controlling factor in the excretion of sodium in the present experiments is not the sodium concentration but the effective osmotic pressure of the serum. However, since urea elevates the serum osmotic pressure without augmenting sodium excretion, it is likely that the effective osmotic pressure of serum functions as a regulatory mechanism, not directly, but by controlling the state of cellular hydration. Thus the failure of urea to sweep out sodium could, on this view, be attributed to the practically unaltered distribution of water between cells and extracellular fluid. Cellular dehydration does, in fact, accelerate the excretion of potassium and it may be that both the excretion of sodium and potassium are in part controlled by the state of hydration of the body cells. There must, however, be counterbalancing influences, since sodium excretion is minimal during water and salt deprivation when the osmotic pressure of the serum and cellular dehydration are increasing.

In contrast to this emphasis on internal regulatory factors, the diuresis of salt which follows the administration of solutes has been attributed solely to the osmotic influence of the administered solute within the renal tubule (5, 6, 29). A number of observations in the literature and in the present study militate against this hypothesis. First, certain solutes, though excreted in amounts comparable to glucose or mannitol, do not elicit the same renal response. Sodium sulfate, an extremely potent diuretic, suppresses the excretion of chloride and produces no increase in sodium excretion beyond that accounted for by the sulfate ion, so that the excretion of sodium chloride is strikingly at variance with that during comparable glucose or mannitol diuresis (30). The failure of urea, noted in these experiments and elsewhere, to promote an overall increase in sodium excretion is similarly unexplained. Second, if mannitol or glucose, by restraining the reabsorption of water in the proximal tubules, impedes the reabsorption of sodium (5, 6, 29) the excretion of other ions should, on purely osmotic grounds, be similarly affected. But in the present study, the excretion of potassium and phosphorus varied comparatively slightly, at times actually decreasing, notwithstanding marked increases in the excretion of sodium. These disproportionate changes in the excretion of different electrolytes are also apparent in the data of Wesson, Anslow, and Smith. Finally, there is ample evidence that the urinary losses of salt during solute administration are not rigidly

determined by the excretion of the injected solute, but vary with the previous state of the organism. This has been demonstrated in the case of glucose by such observation as the lack of correlation between chloride excretion and prolonged glycosuria in diabetics (31), and the failure of a rapidly-induced profuse glycosuria to maintain an accelerated excretion of sodium (32) or to inhibit for any length of time the sparing of sodium in the maintenance of acid-base equilibrium during diabetic acidosis (33). Conflicting observations on urea, demonstrating that it has little effect on sodium excretion (2, 34, 35), that it definitely augments it (4, 36, 37) or that an initial chloruresis is followed by a suppression of chloride excretion (38), may be due to variations in the state of the organism.

Just as renal osmotic considerations do not explain the excretion of salt during a solute diuresis, so the theory of an osmotic ceiling cannot adequately account for the increased urine flow. Shannon pointed out that the concentrations of solutes traversing the renal tubules were not responsible for a glucose diuresis, since the osmotic pressure of the urine during high rates of urine flow fell considerably below the maximum (29). This was confirmed by McCance and his co-workers (37, 39-41). Nevertheless, they ascribed the diuresis to the limiting osmotic pressure of the tubular urine, contending, however, that as urine flow increased, this limiting or maximal value fell. During hydropenia, two consequences follow from this theory: 1) the osmotic pressure of the urine at any given urine flow should be the same, irrespective of the nature of the urinary solute; 2) an inverse relationship between osmotic pressure and urine flow should prevail. There is evidence, however, that when urea is the major urinary solute, less water is excreted at a given urine flow than when other solutes predominate (2, 34, 42-44), and data presented here support this view. Moreover no consistent inverse relationship between urine flow and osmotic pressure during hydropenia is apparent in the data of Ladell (45) or Gamble (46), when sea-water was ingested during rigorous water deprivation. The character of the solute and the extent of dehydration influence the excretion of urine in a manner not explained by its osmotic pressure. The relationship between osmotic pressure and urine flow existing under certain circumstances may be merely a passive result of the particular experimental conditions rather than an expression of the intrinsic limitations of the kidney's ability to perform osmotic work.

The behavior of potassium during these experiments emphasizes the importance of cellular transfers, as well as urinary excretion, in the maintenance of a constant serum concentration (47). Since the excretion of potassium remained constant or increased, while extracellular volume expanded, it follows that accelerated transfer of cellular potassium prevented a fall in the serum concentration. Similarly, contraction of extracellular volume while urinary losses of potassium were constant did not increase the serum concentration because the release of potassium from cells was retarded. Only glucose consistently reduced the concentration of serum potassium. This cannot be ascribed to urinary losses or to changes in extracellular volume, since mannitol did not depress the serum potassium, notwithstanding otherwise comparable effects. Positive intracellular balances, of considerable magnitude in one instance, consistently followed glucose injections. The fall in the serum concentration of potassium following glucose is therefore the result of a movement of potassium

into cells which disturbs the equilibrium normally preserving a fairly constant concentration of potassium when extracellular volume expands. This analysis is consistent with Fenn's data for the rat (48) and with the mechanism of reduction in serum potassium produced in insulin (49).

SUMMARY AND CONCLUSIONS

Hypertonic solutions of glucose, mannitol, and urea were injected into normal human subjects. Glucose and mannitol appear to be confined largely to the extracellular fluid; by elevating the effective osmotic pressure of the serum, they depress the concentration of serum sodium and withdraw water from cells. Both substances sharply augment sodium excretion, whereas urea, excreted in comparable amounts, produces only minor changes. It is concluded from this and other evidence that the state of hydration of body cells may be a regulatory factor in the excretion of sodium.

Injections of hypertonic solutions produce a slight increase in potassium excretion during dehydration and a slight decrease during water ingestion. Cellular transfers of potassium tend to compensate for changes in the concentration of serum potassium which might otherwise result from changes in extracellular volume. The excretion of phosphorus is depressed by mannitol and accelerated by glucose. Urea commands less water for its excretion than do glucose or mannitol.

It is concluded that the effects of hypertonic solutions on the metabolism and excretion of water and electrolytes are not determined by the osmotic pressure of the tubular urine, but depend instead upon the metabolic and osmotic properties of the administered solute, the character and distribution of the electrolyte, and the previous state of the organism.

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PROPORTIONAL CHANGES IN RENAL TUBULAR REABSORPTION OF DEXTROSE AND EXCRETION OF P-AMINOHIPPURATE WITH CHANGES IN GLOMERULAR FILTRATION¹

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SINCE the original work introducing the concept of maximal tubular transfer and describing the constancy of renal tubular reabsorption of glucose at high plasma levels (1, 2), it has generally been assumed that tubular transfer maxima for glucose, diodrast and p-aminohippurate are independent of glomerular filtration rate (3-5). This concept is based on the widely accepted belief that all capillary networks of the nephrons, unlike most capillary beds, are continuously perfused. Accordingly, since all nephrons are continuously supplied with blood, the reabsorbing and excreting mass of the renal tubules is constant and that changes in these functions may only be accomplished by glomerular or tubular damage, inhibition of the transporting enzyme systems or mutual interference. The mammalian kidney, including that of man, is thought to contrast sharply with the amphibian kidney in which Richards and Schmidt showed that nephron intermittency is the rule (6).

During the course of some observations on the effect of diuretics on tubular transfer mechanisms, it became apparent that changes in glomerular filtration rate induced by hydration or dehydration were associated with nearly proportional changes in tubular transport (7). The following report is concerned with the effect of varying glomerular filtration on tubular transport of dextrose and p-aminohippurate.

METHODS

Trained, female dogs weighing between 15 and 26 kilograms were used. In some experiments light anesthesia with pentobarbital sodium was employed. Increases in glomerular filtration rate were effected by infusing large quantities of 0.9 per cent sodium chloride and dehydration by diuretics, or the infusion of 20 per cent dextrose solution, or a combination of both. Glomerular filtration (GF) was measured by creatinine. The maximal rate of tubular transport of dextrose (TmG) and of p-aminohippurate (TmPAH) was measured in independent experiments, because of the reported mutual interference in the transfer of these two substances (8,9). The plasma concentration ranges maintained were as follows: creatinine 20 to 50 mg. per cent; dextrose 500 to 800 mg. per cent; p-aminohippurate (Tm) 40 to 100 mg. per cent and 1 to 2 mg. per cent for measuring renal plasma flow (RPF). A priming dose of these substances was administered followed by constant intravenous infusion of the mixture. Infusion rates were changed following hydration or dehydration, in accordance with anticipated changes in glomerular filtration, before a new series of clearances was determined. This was done to prevent excessive blood levels of glucose or concentrations too low for Tm measurement, depending on whether glomerular filtration was experimentally being decreased or increased.

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Cadmium sulfate filtrates were used for all plasma determinations. Hanes' (10) modification of the Hagedörn Jensen method was employed for both plasma and urine glucose. The standard methods for creatinine and p-aminohippurate were used. Blood samples were taken two minutes before the mid-point of each clearance period. The general procedure adopted for these experiments was to make control measurements, immediately produce hydration or dehydration and repeat the observations. In some experiments dehydration was produced after hydration (table 4).

RESULTS

The effects of moderate dehydration, produced by several hours of mercurial or theophylline diuresis, on glomerular filtration and tubular reabsorption of dextrose are indicated in table 1. These diuretics do not have any direct effect on glomerular filtration or tubular transport of dextrose or p-aminohippurate in the dog (7, 11). Kidney function is not altered at the peak of diuresis, which occurs at approximately one hour, but only after the urine output begins to decline, and sufficient water has been eliminated to produce moderate dehydration. These effects usually become apparent after about two hours of diuresis, but their magnitude increases with time and the amount of water eliminated. It is evident from table 1 that the ratio, glomerular filtration to TmG, remains nearly constant when moderate changes in glomerular filtration are produced.

In table 2, the initial set of figures represents the control values for GF, TmG and RPF. The second set of measurements was made after the infusion of 500 cc. to one liter of 20 per cent dextrose at a rate of 10 to 15 cc. per minute. Several hours were usually allowed to elapse after the dextrose infusion as indicated in table 4. The results show that the TmG and renal plasma flow decrease as glomerular filtration falls. The ratio, GF to TmG, decreases with the more severe degrees of dehydration due to hemoconcentration and a fall in the filtered fraction (FF). *Dog Tm-10* (table 2) shows the magnitudes of the changes in renal function that may be produced by severe dehydration. This animal died about 5 hours after the termination of the experiment, from excessive water loss as has been noted by others (12, 13).

Table 3 shows the effect of changing GF on the tubular excretion of p-aminohippurate. Again it is apparent that there is a parallel relationship between GF rate and tubular transport, whether GF is increased by the rapid infusion of a large quantity of saline or decreased by dehydration. Table 4 shows the data on an individual dog in which both hydration and dehydration have been produced.

DISCUSSION

There are several conceivable explanations for these results such as: 1) an effect of glomerular filtration on the tubular transporting mechanisms; 2) an effect of cellular hydration or dehydration on the tubular transfer mechanisms; and 3) a change in the number of active nephrons. Although these experiments offer no critical data on the mechanism involved, we believe that a changing number of active nephrons is the most likely explanation. The relatively constant value for the ratio GF to TmG is in harmony with this explanation. The rate of reabsorption of bicarbonate and chloride has been observed to be nearly proportional to glomerular filtration rate (14, 15) when glomerular filtration was varied by meat feeding or the infusion of alanine. Although renal plasma flow, TmG or TmPAH were not measured,

it seems not unlikely that the direct relationship between glomerular filtration and tubular transport observed for these substances is again a reflection of the number of active nephrons.

Measurements of glomerular filtration, TmG and TmPAH have demonstrated that these functions of the kidney remain constant in both animals and man when

TABLE 1. EFFECT OF MODERATE DEHYDRATION ON GLOMERULAR FILTRATION AND RENAL TUBULAR REABSORPTION OF DEXTROSE

DOG NO.	WT.	GF ¹	TmG ¹	GF TmG	% CHANGE		METHOD OF DEHYDRATING
					GF	TmG	
Tm-2	17	82	283	0.29			Control
		54	208	0.26	34	26	0.1 cc/kg. Mercuhydrin
-2	17	84	268	0.31			Control
		67	234	0.29	20	13	20 mg/kg. Theophylline
-3	16	64	270	0.24			Control
		43	184	0.23	33	32	20 mg/kg. Theophylline
-4	15	73	265	0.28			Control
		61	220	0.28	16	17	0.1 cc/kg. Mercuhydrin
-5	19	64	280	0.23			Control
		40	174	0.23	37	38	0.1 cc/kg. Mercuhydrin
-5	19	60	235	0.26			Control
		44	160	0.27	27	32	0.1 cc/kg. Mercuhydrin
-6	14	62	195	0.32			Control
		36	150	0.24	42	23	0.2 cc/kg. Mercuhydrin
-6	14	55	211	0.26			Control
		34	145	0.23	38	31	0.2 cc/kg. Mercuhydrin
-7	17	60	232	0.26			Control
		47	175	0.27	22	25	0.1 cc/kg Mercuhydrin

¹ Each figure represents an average of three consecutive 10-minute periods.

repeated determinations are made over long periods of time. With the standard procedures for making these measurements, a fairly uniform degree of hydration is produced to insure an adequate urine flow, and therefore presumably a nearly constant number of active nephrons. Even under these circumstances, we do not believe all nephrons are active, since it is possible to increase glomerular filtration with a proportional increase in TmG and TmPAH (table 4). Changes in hemodynamics of the kidney may, in fact, be induced by the usual methods employed for determining Tm's. We have observed that in the dog glomerular filtration usually is considerably greater when Tm's are measured than when glomerular filtration is determined alone

TABLE 2. EFFECT OF DEHYDRATION 20 BY PER CENT DEXTROSE SOLUTION ON GLOMERULAR FILTRATION, RENAL PLASMA FLOW AND RENAL TUBULAR REABSORPTION OF DEXTROSE

DOG NO.	WT.	DATE	GF ¹	TmG ¹	RPF (PAH)	TF	GF TmG
	kg.		cc/min.	mg/min.	cc/min.		
Tm-1	17	4-19-49	60	205	223	0.27	0.29
			44	175	185	0.24	0.25
-1	17	5-3-	65	198	211	0.31	0.33
			29	122	97	0.30	0.24
-3	15	2-25-	50	185	190	0.26	0.27
			34	142	124	0.27	0.24
-3	15	3-17-	60	236	153	0.39	0.25
			32	127	85	0.38	0.25
-8	19	4-20-	58	214	171	0.34	0.27
			41	158	140	0.29	0.26
-9	22	4-22-	80	254	271	0.29	0.31
			41	162	220	0.19	0.25
-9	22	5-9-	76	240	335	0.23	0.32
			30	155	140	0.19	0.21
-10	22	4-18-	48	152	200	0.24	0.32
			9	70	45	0.20	0.13

¹ Each figure represents an average of three consecutive 10-minute periods.

TABLE 3. EFFECT OF HYDRATION AND DEHYDRATION ON GLOMERULAR FILTRATION AND TUBULAR EXCRETION OF P-AMINOHIPPURATE

DOG NO.	WT.	GF ¹	TmPAH ¹	METHOD OF CHANGING GLOMERULAR FILTRATION
	kg.	cc/min.	mg/min.	
Tm-1	17	60	18	Control
		78	24	Infusion of 0.9% saline
-6	14	68	14	Control
		36	8	Infusion of 20% dextrose
-9	22	61	11	Control
		88	19	Infusion of 0.9% saline
-12	20	40	13	Control
		15	5	Infusion of 20% dextrose and 0.1 cc/kg. Mercuhydrin
-13	26	70	32	Control
		33	9	Infusion of 20% dextrose

¹ Each figure represents an average of three consecutive 10-minute periods.

with no intravenous infusion. When dextrose solutions are infused, glomerular filtration and the TmG will remain constant for prolonged periods after the hemodynamics of the kidney have stabilized, provided the plasma dextrose level does not become excessive. If the dextrose level approaches one per cent, within one or two hours glomerular filtration, renal plasma flow and the TmG fall considerably due to dehydration. These factors may be involved in the reported mutual interference of the maximal rate of transfer of dextrose and p-aminohippurate (9, 11).

In view of these observations, it is probable that the interpretations of certain data reported in the literature (16, 17) should be reconsidered. This is particularly true of experiments involving the administration of thyroxine, which in our experience

TABLE 4. EFFECT OF HYDRATION AND DEHYDRATION ON GLOMERULAR FILTRATION, TmG AND RENAL PLASMA FLOW

Dog Tm-9, 22 kg.

TIME	GF	TmG	$\frac{GF}{TmG}$	RPF (PAH)	F.F.
min.	cc/min.	mg/min.		cc/min.	
<i>Control</i>					
0-10	63	204	0.31	242	0.26
10-20	66	210	0.31	238	0.28
<i>Infusion of 1 liter of 0.9% sodium chloride 50 cc/min.</i>					
32-42	76	254	0.30	335	0.23
42-52	79	240	0.33	335	0.24
52-62	72	220	0.33	340	0.21
<i>Infusion of 600 cc. of 20% dextrose 10 cc/min.</i>					
364-74	33	156	0.21	176	0.19
374-84	31	165	0.19	190	0.16
384-94	28	146	0.19	160	0.18

increases glomerular filtration, renal plasma flow and TmG without appreciably altering the filtration fraction (18).

The data indicate that the kidney of the dog is capable of considerable hemodynamic changes in response to changes in body water and that activation or inactivation of nephrons is an additional mechanism for conserving or facilitating the elimination of water.

SUMMARY

Moderate dehydration causes a decrease in glomerular filtration, renal plasma flow and glucose and p-aminohippurate Tm's without appreciably changing the filtration fraction. These effects become more prominent as dehydration progresses. Excessive hydration produces the reverse effects. Dehydration is believed to inactivate, hydration to increase the number of active nephrons. This mechanism is believed to be an additional mechanism for maintaining fluid balance in the dog.

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CARINAMIDE (4'-CARBOXYPHENYLMETHANESULFONANILIDE)¹: ITS RENAL CLEARANCE AND BINDING ON PLASMA PROTEIN

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IT HAS been reported that carinamide, 4'-carboxyphenylmethanesulfonanilide, inhibited selectively the excretion of penicillin by the renal tubules (1); p-aminohippurate and phenol red were excreted by the same tubular transport mechanism as penicillin and likewise their excretion was inhibited (2). The hypothesis that led to the development of carinamide predicted that this could be done by a compound that was well absorbed on oral administration, that did not inhibit the reabsorption of essential metabolites, and that was essentially refractory to excretion by the tubules (3).

Carinamide has been found to fulfill these requisites insofar as they have been tested (1-4). It has proven to be of value as an adjunct to penicillin therapy due to the elevation of antibiotic blood level and the prolongation of its retention in the body resulting from the suppression of penicillin excretion by the renal tubules (5). The selectivity of carinamide for this particular tubular excretory mechanism has been substantiated recently by the finding that the drug does not inhibit the tubular excretion of N-methylnicotinamide. This latter compound is excreted by a tubular mechanism distinct from that which excretes penicillin, p-aminohippurate and phenol red (6).

The purpose of the research reported herein has been to study the renal clearance of carinamide and its metabolite, and their binding on, and diffusion from, plasma protein. A preliminary report by Earle and Brodie (7) has indicated that a portion of carinamide was bound on plasma protein, and that the clearance ratio for the drug was about 0.50, as compared to creatinine in the dog. When calculated on the basis of the plasma concentration of unbound drug, the clearance varied in their experiments from a value equal to glomerular filtration rate to one considerably exceeding glomerular filtration rate.

A broader basis for a more critical consideration of the excretion of carinamide and its metabolite is presented herein, as drawn from repeated simultaneous renal clearances of the compounds in several types of experiments with the determination of drug concentration in whole plasma and plasma water. Twelve trained clearance dogs have been used in this series of experiments, and another group of 8 dogs having explanted kidneys were used in another related research (8).

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¹'Staticin' is the trademark applied to 4'-carboxyphenylmethane-sulfonanilide, carinamide by Sharp & Dohme, Inc. This compound has been referred to in previous literature as caronamide.

METHODS

We have used two methods for the determination of carinamide and its metabolite throughout this research. The method of Brodie, Levy and Bernstein (9) has been used to measure carinamide per se. This it does satisfactorily when the drug is added to plasma or urine (9, 2). Present chromatographic and countercurrent distribution studies confirm the specificity of the test in metabolism studies. We have discussed the method in more detail in previous reports (2, 4a).

The method of Ziegler and Sprague (10) measures carinamide and its metabolite (*total carinamide*) when the compound is administered to man or other animals. Although it determines satisfactorily carinamide added to urine or plasma it does not distinguish between this agent and its more water soluble metabolite. Since by this method the percentage urinary recovery of a given dose of carinamide is complete, on an average (4a) it probably measures the drug and its metabolite. These have been referred to herein and in our previous reports as *total carinamide*, since we do not know the identity of the metabolic product. We have discussed the method in more detail previously (2, 4a). In order to minimize any analytical error we have prepared a standard curve for carinamide at the time of each experiment. This precaution is not necessary for clinical interpretation since the variation in the slope of the curve is not marked.

It is important to emphasize the rigorous attention to technical details in the actual clearance procedure and chemical analyses. Even so the data vary considerably probably in part through differences in the quantity and identity of metabolite(s) formed which may have somewhat different clearances or for which the analytical conditions may not be ideal. For example, the glycine conjugate of carinamide is determined qualitatively by either analytical method but neither test permits a precise quantitative determination of the compound when performed as described by the authors.

Creatinine clearances were performed as a measure of glomerular filtration rate in the dog (11). Creatinine was determined by the alkaline picrate method of Folin following trichloroacetic acid precipitation of protein.

The determination of carinamide and *total carinamide* in plasma water was performed on the plasma ultrafiltrate obtained by the procedure of Lavietes (12), using a specially treated no. 300 moisture-proof cellophane membrane and a pressure of approximately 35 cm. of Hg. This direct method for the determination of unbound or ultrafilterable drug has been most satisfactory and accurate in our hands. However, there is no reason to believe that this cellophane membrane and these conditions simulate the conditions of glomerular filtration any more adequately than the diffusion-dialysis method described for the measurement of binding. The ultrafiltration method of Lavietes together with the determination of plasma albumin electrophoretically and the estimation of total protein by a modification of the micro method of Howe (13) permitted the precise calculation of the adsorption isotherms of carinamide and *total carinamide* presented herein. Also, insofar as they were applicable to the interpretation of our results, it was felt that it would be most desirable to determine the actual amount of drug in the ultrafiltrate of a given plasma sample rather than to make indirect estimates from adsorption or binding curves obtained on other plasmas.

The determinations of p-aminohippurate (PAH) have been performed by the method of Smith, *et al.* (14). It was found that PAH was not detectable by the method of Brodie *et al.* nor did it interfere with the determination of the recovery of carinamide therapy. On the other hand PAH was detectable by the method of Ziegler and Sprague because of the presence of the aromatic amino group in the molecule. In our preliminary recovery experiments it was found that at various plasma and urine concentrations an average of 90 per cent of PAH could be recovered when determined alone or when added to a known concentration of carinamide in solution. Consequently, to determine the amount of *total carinamide* in a plasma or urine sample containing PAH a value representing 90 per cent of the PAH content, as determined by the Smith method, was subtracted from the total Ziegler-Sprague value for the same sample. However, we have omitted the *total carinamide* data from the PAH experiments because of our present lack of assurance as to their accuracy. PAH and creatinine have always been found by us in repeated experiments to be totally ultrafilterable according to the method of Lavietes. This simplified somewhat the complicated determination of carinamide, *total carinamide*, PAH and creatinine in whole and ultrafiltered plasma samples from each experiment. In most of the clearance calculations the plasma determinations were performed on arterial samples, although the use of plasma from blood drawn simultaneously from the opposite femoral vein for the determination of clearance introduced no consistent or significant alteration in the comparative results.

The experiments were designed as simply as possible to minimize the number of bleedings, because of the large amount of blood removed per sample and the considerable analytical effort expended on each clearance period. The details of protocols are presented with the consideration of each type of experiment.

RESULTS

The binding of carinamide and total carinamide on plasma protein may be considered appropriately here since the general consideration of this subject will carry through the discussion of the clearances.

The protocol for the experiments from which the isotherm data were obtained was as follows: After the initial removal of a sample of blood for the determination of control values the dogs were given a dose of 0.5 gm. of carinamide subcutaneously. Thirty minutes later a sample of blood adequate for all the necessary analyses was

TABLE 1. ADSORPTION ISOTHERM DATA OBTAINED FOR CARINAMIDE AND TOTAL CARINAMIDE FROM PLASMA OF DOG 441 ACCORDING TO PROTOCOL AND METHODS DESCRIBED IN THE TEXT

SAMPLE NO.	PLASMA CONC. MG/100 CC.	LOG ¹ PLASMA CONC.	MG. BOUND	GM. ALBUMIN	MG. BOUND ² GM. ALBUMIN	LOG ³ MG. BOUND GM. ALBUMIN
<i>Carinamide</i>						
1	2.51	0.3996	1.48	2.132	0.694	-1.8413
2	9.08	0.9580	4.43	2.129	2.080	0.3180
3	26.49	1.4230	12.53	2.125	5.894	0.7704
<i>Total Carinamide</i>						
1	3.64	0.5611	1.98	2.132	0.928	-1.9677
2	12.48	1.0962	6.34	2.129	2.977	0.4737
3	33.90	1.5302	16.12	2.125	7.583	0.8798

¹ = log c. ² = \bar{x}/m . ³ = log \bar{x}/m , expression of the coordinates for fig. 1.

drawn. The dogs then were given 1.5 gm. of carinamide subcutaneously and a second blood sample was taken 30 minutes later. Three grams of the drug then were injected subcutaneously and after an interval of 30 minutes a blood sample was taken. The protocol was modified somewhat to obtain more intermediate values and a greater range of plasma concentrations in various experiments. To enhance the accuracy of the results albumin and total protein determinations were performed on each sample wherein that was feasible.

In the conventional expression of an isotherm the logarithm of the amount of a drug bound per gram of adsorbent (albumin) is a linear function of the logarithm of its plasma concentration at all but exceedingly high levels, if one may translate freely the usual physical terminology into physiological terms. A summary of the data for carinamide and *total* carinamide obtained according to the protocol and methods described are presented in table 1. To demonstrate the adequacy with which they fit the conventional expression of an isotherm the data from table 1 have been plotted in figure 1.

Carinamide and its metabolite(s) were bound on albumin in accordance with

the concepts of physical adsorption isotherms, as evidenced by these and confirmatory data. Interestingly, a single curve represented adequately the data in this and most other confirmatory experiments for both the Brodie and the Ziegler-Sprague determinations of the drug and its metabolite(s). At a given plasma concentration the adsorption of carinamide and *total* carinamide were the same (fig. 1).

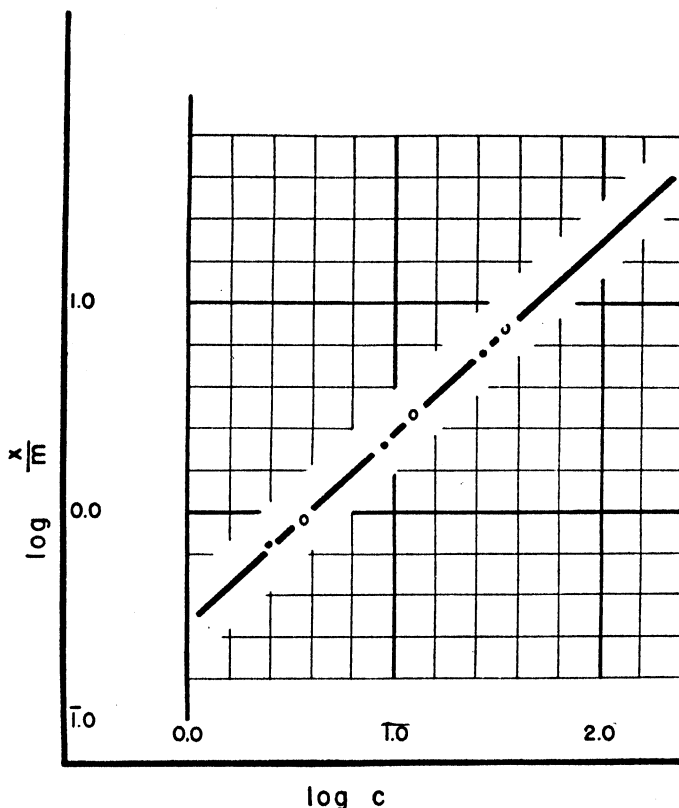


Fig. 1. AN ISOTHERM for carinamide (—●—) and *total* carinamide (—○—) adsorption on plasma protein. Carinamide was administered to the dog and blood was withdrawn periodically. The amount of drug bound per gram of albumin (x/m) and the concentration in whole plasma (c) were determined. The data from which this figure was prepared are contained in table 1.

These data may be plotted to give the percentage binding per unit of albumin or total protein as a function of plasma concentration. However, the absolute values for the amount of drug bound at any given plasma concentration depends in part on the concentration and the binding characteristics of the adsorbent per se and as influenced by variations in the concentrations of other constituents. For example, Reinhold, Flippin, Domm and Pollack reported that the amount of certain sulfonamides bound per unit of albumin at a given plasma concentration varied from patient to patient and with the severity and the nature of the illness (15). Thus where precise determinations of drug concentration in plasma water are desirable the safest procedure would seem to be one of ultrafiltration of a given plasma sample.

Lavietes has discussed other theoretical aspects of the procedure (12), and Davis recently has reviewed this field admirably (16).

The desorption or diffusion of carinamide from plasma protein can be demonstrated to occur readily. In these experiments a dose of carinamide (2-5 gm.) was administered intravenously to dogs following the removal of sufficient blood (about 40 cc.) to supply plasma for the control spectrophotometer settings in the experiment. Approximately 20 minutes after the injection of carinamide 80 to 90 cc. of blood were withdrawn and the plasma removed therefrom. The carinamide and *total* carinamide content of the whole and ultrafiltered plasma sample obtained after drug administration was determined as a control value at zero time. The two samples of plasma then were poured into Visking (cellophane) tubes one-half inch in diameter and suspended individually in 19.96 liters of Kreb's Ringer-phosphate solution containing potassium oxalate. The solution was not changed and was stirred at a constant rate at room temperature. Samples of plasma were removed from both of the

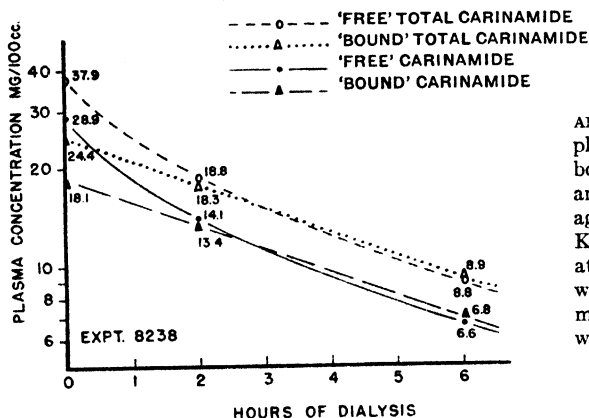


Fig. 2. AMOUNT OF CARINAMIDE and *total* carinamide in plasma water and calculated as bound on plasma protein before and at intervals following dialysis against approximately 20 liters of Kreb's Ringer-phosphate solution at room temperature. Carinamide was administered to the dog 20 minutes before the plasma was withdrawn.

bags over a period of 6 hours for the determination of carinamide and *total* carinamide. The volume of plasma was such that at least 5 to 10 cc. remained in the cellophane tubes at the termination of the experiment.

The results of such an experiment are plotted in figure 2. It may be seen that the diffusion of both free and bound drug takes place rapidly and to an extent greatly exceeding the amount determined to have been ultrafilterable before dialysis. Because of the simplicity of the diffusion experiments they have been intended only to determine qualitatively the desorption and diffusion characteristics of the drug. It seems clear that carinamide bound on plasma protein is capable of desorption from the bound state and of diffusion through a permeable membrane.

The facts that carinamide 1) is adsorbed on plasma proteins in accordance with the adsorption isotherm principle and 2) diffuses readily from this bound state conform with the principal characteristics of physical adsorption, as opposed to chemical reaction. Since physical adsorption is the concentration of atoms or molecules at surfaces or interfaces in a readily alterable fashion it follows that these accumulated molecules are 'shared' at the interface by the adsorbent (protein) and solvent (plasma water). Thus any alteration in the equilibrium between protein and

plasma water involving a shift in their relative concentrations, as by ultrafiltration at the glomerulus or by changes in electrolyte balance, could be expected to influence simultaneously the distribution of a compound between the interface and the solvent.

The renal clearances of carinamide and total carinamide were performed in a series of some 60 or so experiments on 20 well trained unanesthetized dogs. In general 1 to 3 gm. of carinamide were administered subcutaneously or the drug was infused continuously intravenously to maintain a uniform plasma concentration in the manner of the experiments on the inhibition of penicillin excretion (1). One-half hour after the drug was administered one to three 10-minute clearances were performed, an oxalated blood sample being taken at the midpoint of each period. The plasma determinations of carinamide and *total* carinamide were performed on whole plasma and (ultrafiltered) plasma water. The carinamide or *total* carinamide/creatinine clearance ratios and the corresponding 'excretion' ratios were calculated. The 'excretion' ratio is actually a clearance ratio wherein the carinamide or *total* carinamide clearance is calculated on the basis of the drug concentration in plasma water. Creatinine is not bound on plasma protein and is freely ultrafilterable. We shall refer to the 'excretion' ratio herein as the 'corrected' clearance ratio, meaning that the clearances have been calculated on the basis of the unbound concentration of drug in plasma water. The propriety with which either term 'excretion' ratio or 'corrected' clearance ratio is used in this report as it pertains to carinamide or *total* carinamide will be deferred to the discussion of results.

In table 2 we have summarized the data on 5 experiments performed on each of 4 dogs, since they are representative of the group as a whole. In table 2 the experiments on each dog have been arranged, generally, on the basis of increasing plasma concentration of carinamide.

The clearance of carinamide and *total* carinamide decreased with increasing plasma concentration (table 2). This was the dominant characteristic of the results and of course held for both the 'corrected' and 'uncorrected' clearance ratio. Although the absolute value of the clearance for either determination of the drug fluctuated with urine flow and creatinine clearance, within and between experiments on a given dog, the clearance ratio remained reasonably constant where there was no considerable difference in the drug plasma concentration.

Within any given experiment the plasma concentration of *total* carinamide was of course greater than that of carinamide per se, and the clearance ratio for *total* carinamide usually was less than that for the drug per se. The lower clearance ratio for *total* carinamide in a given experiment does not necessarily imply a difference in renal elimination. A comparison of carinamide and *total* carinamide clearance ratios at similar plasma concentrations in different experiments on the same dog will show them to be essentially the same, or to differ in one direction or the other unpredictably.

The percentage of ultrafilterable drug did increase with rising plasma concentration but not as systematically as would be anticipated from the isotherm (fig. 1), due to the fact that the experiments were run on different days at which time the amount and character of the adsorbent or other constituents that might influence

TABLE 2. SUMMARY OF RENAL CLEARANCE DATA¹ FOR CARINAMIDE A AND TOTAL CARINAMIDE B AS REPRESENTED BY EXPERIMENTS ON 4 DOGS

EXPER. NO.	CREATININE CLEARANCE	URINE FLOW	PLASMA CONC.	AMOUNT EXCRETED	CLEARANCE	CLEARANCE RATIO	PERCENTAGE ² ULTRA-FILTERABLE	'CORRECTED ³ CLEARANCE'	'CORRECTED ³ CLEARANCE' RATIO
<i>Dog 84</i>									
	<i>cc/min.</i>	<i>cc/min.</i>	<i>mg/100 cc.</i>	<i>mg/min.</i>	<i>cc/min.</i>				
9107	89.7	6.5	A 6.3 B 11.0	4.38 6.17	69.5 56.1	.77 .63			
9127	93.7	6.8	A 6.7 B 11.5	4.00 6.23	65.7 54.2	.69 .58			
1208	52.6	0.7	A 7.7 B 10.8	3.0 3.51	38.8 32.5	.74 .62	39 38	99.9 85.7	1.90 1.63
1247	73.6	0.4	A 18.5 B 25.4	5.29 6.78	28.5 26.7	.39 .36	55 37	52.2 42.3	0.71 0.58
11197	54.1	0.5	A 24.4 B 44.0	2.83 4.68	11.5 10.6	.21 .20	54 43	21.3 24.6	0.39 0.46
<i>Dog 365</i>									
9167	67.3	5.0	A 4.3 B 9.5	2.57 4.25	59.1 44.7	.88 .66			
9107	64.1	4.8	A 7.3 B 11.5	3.23 3.60	44.0 31.3	.69 .49			
1157	73.9	5.6	A 7.2 B 18.5	2.16 4.65	29.8 25.2	.40 .34	43	71.2	0.96
178	58.3	3.5	A 10.8 B 21.1	2.88 3.64	26.5 17.2	.46 .30	46 60	58.0 28.8	0.99 0.50
12297	46.7	3.3	A 16.3 B 30.2	3.08 4.49	18.8 14.8	.40 .32	46 62	41.1 22.8	0.88 0.51
<i>Dog 285</i>									
1148	72.7	1.8	A 5.0 B 7.8	3.30 3.23	66.1 41.4	.91 .57	34 32	194.5 194.5	2.67 2.67
10317	84.2	4.6	A 7.5 B 16.5	5.57 5.75	74.3 34.8	.88 .41	50	150.0	1.71
4297	70.4	0.7	A 6.9	2.82	40.5	.58			
11127	67.5	2.8	A 11.8 B 24.0	3.61 5.82	30.4 24.2	.45 .36	45	67.6	1.00
12167	81.3	0.5	A 11.2 B 17.8	5.10 7.40	35.7 41.5	.44 .51	49 56	73.5 74.0	0.90 0.91

TABLE 2—Continued

EXPER. NO.	CREA- TININE CLEAR- ANCE	URINE FLOW	PLASMA CONC.	AMOUNT EX- CRETED	CLEAR- ANCE	CLEAR- ANCE RATIO	PER- CENTAGE ² ULTRA- FILTER- ABLE	'COR- RECTED' ³ CLEAR- ANCE'	'COR- RECTED' ³ CLEAR- ANCE' RATIO
Dog 370									
1138	81.4	4.4	A 5.0	3.69	73.2	.90	30	244.6	3.00
			B 8.3	3.98	47.9	.59	28	173.1	2.13
1278	69.9	5.0	A 4.7	2.92	61.2	.88	53	86.8	1.6
			B 7.6	2.87	37.7	.54	38	91.4	1.68
10247	79.2	5.4	A 6.3	4.39	69.0	.87	67	103.2	1.3
			B 15.8	7.05	44.6	.56			
778	70.8	1.0	A 17.4	5.46	31.2	.44	42	74.3	1.05
			B 24.0	6.25	25.9	.37	48	54.1	.76
6238	77.4	0.5	A 14.1	6.19	25.2	.33	43	58.2	0.75
			B 20.3	4.50	22.0	.29	43	50.8	0.66

¹ All data are based on data calculated to the second decimal place. ² 'Corrected clearance' actually represents the clearance calculated on the basis of *in vitro* ultrafilterable (12) plasma concentration. ³ This is also referred to in the text as the excretion ratio.

adsorption may have been different. The agreement between ultrafiltrate determinations on successive plasma determinations has been quite satisfactory.

Since the absolute value for carinamide clearances, or *total* carinamide clearances, decreased with increased plasma concentration, the clearance ratios and 'corrected' clearance ratios decreased similarly (table 2). There are two striking and unusual features about the decrease in these ratios. First, in the whole series of experiments, of which 21 are represented in the table, there was no experiment in which the carinamide/creatinine clearance ratio for carinamide or *total* carinamide was reproducibly greater than 1.0. At elevated plasma concentration the clearance ratio was reduced to as low as 0.20 for carinamide and 0.15 for *total* carinamide. Secondly, the 'corrected' clearance ratio for carinamide and *total* carinamide varied from values greater than 2.0 in some instances at low plasma concentration to as low as 0.42 at elevated plasma concentrations. This is an unusual circumstance that could not have been anticipated by Earle and Brodie in their single experiments on each of 3 dogs, although their average ratios for each of the experiments showed a considerable variation (7). Further consideration of these results will be given in the DISCUSSION.

The effect of *p*-aminohippurate on the clearance of carinamide was studied since it was felt that such data might be of aid in the overall evaluation of its elimination. We reported previously that carinamide inhibited the tubular excretion of *p*-aminohippurate (2).

In these experiments the dogs were given water by stomach tube and 3.0 gm.

of creatinine were injected subcutaneously. The dogs were given a single intravenous injection of 25 mg/kg. of carinamide immediately followed by a venoclysis of the drug which continued throughout the experiment at a rate of 30 mg/kg/hr. The compound was infused as the sodium salt, pH 7.4, in 5 per cent glucose at a rate of 3 cc/min. After a half-hour equilibration period one 10-minute control clearance period was performed. Following the control clearance period a single intravenous injection of 100 mg/kg. of PAH was made and the continuous venoclysis was switched to a solution that permitted the infusion of 100 mg. of PAH/kg/hr. in addition to the previous rate of carinamide infusion. After a half-hour period for equilibration an additional 10-minute clearance was performed. Creatinine and PAH determinations were carried out on whole plasma. Carinamide determinations were made on whole and ultrafiltered plasma. Determinations of all the agents were carried out on urine. The protocol just described was altered in some instances to permit higher plasma concentrations of carinamide. The dosage of PAH was selected to give a plasma concentration of at least 25 mg/100 cc. At least 2 experiments were performed on each of 3 dogs. Table 3 summarizes representative experiments on each of the dogs and 3 experiments at different carinamide plasma concentrations in one dog.

The coadministration of PAH decreased the clearance, clearance ratio and 'corrected' clearance ratio of carinamide per se in each experiment. This was true regardless of whether the 'corrected' clearance ratio in the control phase was greater or less than 1.0. Moreover, in all the experiments the absolute value for the amount of carinamide excreted after the infusion of PAH was always less than the amount that could be calculated to have been filtered on the basis of the amount of drug in whole plasma or plasma water. A more adequate interpretation of these data is presented in the DISCUSSION.

DISCUSSION

Any interpretation of the data ultimately must take into consideration these several points: 1) The percentage urinary recovery of carinamide per se over a 4-hour period averaged half that of mannitol, and the total recovery of carinamide plus its metabolite(s) averaged only slightly less than that of mannitol. This was consistent with the observation that 2) the ratio of the slope of the *total* carinamide/mannitol falling plasma concentration was less than 1.0 (av. 0.77) whereas the ratio of the slope of the curve representing excretion plus metabolism of carinamide/mannitol averaged 1.09 (4a). With these data as approximations it was not surprising that 3) the clearance ratios of carinamide and total carinamide/creatinine were essentially the same at equal plasma concentrations and were less than 1.0 at all plasma concentrations. 4) Also, the ratio of the extraction of carinamide or *total* carinamide to that of creatinine from the renal blood stream never was reproducibly greater than 1.0 (8). The interpretation must be consistent with the observations that 5) there was a reciprocal relationship between plasma concentration and the clearance of carinamide or *total* carinamide; that 6) calculated on the basis of unbound drug, the 'corrected' clearance ratios decreased from 1.90 to 3.00 to considerably less than 1.0 (0.39-0.75) with increasing plasma concentration

TABLE 3. EFFECT OF P-AMINOHIPPURATE (PAH) ON RENAL CLEARANCE OF CARINAMIDE

DOG; EXPER. NO.	BEFORE AFTER	URINE FLOW	CREA- TININE CLEAR- ANCE GF	CARINAMIDE							CARINAMIDE CREATININE	
				Concentration in		Amt. filtered ¹		Amt. ex- creted UV	Clearance		Clear. ³ ratio	Ex- cretion ⁴ ratio
				Whole plasma Pt	Plasma ¹ water Pw	GF × Pt	GF × Pw		UV/Pt	UV/Pw		
		cc/min.	cc/min.	mg/100 cc.	mg/100 cc.	mg/100 cc.	mg/100 cc.	mg/min.				
84 4148	B	2.8	63.57	8.20	3.83	5.21	2.43	4.08	49.72	106.60	0.78	1.68
	A	6.4	71.67	9.18	4.49			2.27	24.69	50.49	0.34	0.70
PAH	A ⁵	Tm = 1.59 mg/min.		23.64		16.95		18.54	78.41		1.09	
370 3308	B	4.7	75.92	7.50	3.08	5.69	2.33	3.36	44.88	109.29	0.59	1.44
	A	2.95	67.75	9.76	4.34			0.99	10.15	22.82	0.15	0.33
PAH	A	Tm = 3.21 mg/min.		25.43		17.23		20.45	80.39		1.19	
365 3318	B	1.1	48.32	8.98	3.67	4.34	1.77	2.15	23.94	58.52	0.50	1.21
	A	0.9	68.27	9.00	4.13			1.39	15.43	33.65	0.23	0.49
PAH	A	Tm = 3.00 mg/min.		24.75		16.90		19.90	80.39		1.18	
788	B	2.9	50.70	13.29	5.57	6.74	2.82	3.02	22.74	54.24	0.45	1.07
	A	3.9	52.16	16.05	6.81			1.62	10.07	23.75	0.19	0.46
PAH	A	Tm = 2.28 mg/min.		29.97		14.59		16.87	60.31		1.15	
7128	B	3.1	53.07	23.97	12.21	12.72	6.47	4.32	18.02	35.37	0.34	0.67
	A	1.8	48.39	28.88	18.18			2.96	10.25	18.30	0.21	0.38
PAH	A	Tm = 0.91 mg/min.		27.12		13.12		14.03	51.73		1.07	

¹ *In vitro* ultrafiltrate (12).² Probably neither figure for the amount filtered is absolute. GF × Pt = maximal amount of drug that could be filtered, and GF × Pw = amount filtered if only the percentage calculated as 'unbound' in plasma were available for filtration.³ Clearance ratio calculated on clearance from whole plasma.⁴ Caronamide clearance calculated on the basis of 'unbound' drug in plasma.⁵ A = values for PAH in the second portion of the experiment. Note that even though the Tm is depressed and no binding correction is introduced the clearance ratio for PAH/creatinine is greater than 1.0.

(table 2); and that 7) p-aminohippurate was capable of decreasing the clearance ratio or 'corrected' clearance ratio of carinamide regardless of whether the value for the latter ratio was greater or less than 1.0 in the control phase of the experiment (table 3).

Although the falling plasma concentration curves and the urinary recovery data would indicate that the overall renal elimination of carinamide or *total* carinamide was of the order of glomerular filtration rate, it is evident from the clearance data that more than just filtration of unbound drug is involved. A very substantial reabsorption of carinamide and its metabolite(s) is indicated by the fact that at high plasma concentration or after the coadministration of PAH the drug/creatinine clearance ratios are considerably less than 1.0 regardless of whether they are based on whole or ultrafiltered plasma concentrations. Stated differently, at high plasma concentration or after the coadministration of PAH the amount of carinamide excreted per unit time (UV) is less than the amount that can be calculated to have been filtered at the glomerulus, regardless of whether the whole plasma or plasma water concentrations of the drug are used for the calculations. However, glomerular filtration of unbound drug plus tubular reabsorption of a portion thereof is inadequate to account for the overall picture of carinamide excretion, for at low plasma concentrations the amount of the drug excreted frequently is greater than can be accounted for on the basis of ultrafiltration of only unbound compound from plasma water even if there were no tubular reabsorption.

The amount of drug excreted in excess of that filtered and reabsorbed can be accounted for by the contribution of a portion of so-called 'bound' drug to the amount filtered at the glomeruli, or by both tubular secretion and reabsorption of the drug and its metabolite(s) with only 'unbound' drug being filtered at the glomeruli. Both of these interpretations imply that a maximal reabsorptive capacity for the compounds has not been reached at plasma concentrations of over 20 mg/100 cc., and that the reabsorption of the drug is not influenced demonstrably by the coadministration of PAH (table 3).

If it be supposed that the sole elimination of the drug is by glomerular filtration, the hypothesis that the electrostatic attraction (binding) between the drug and plasma protein behaves broadly as an impedance to its filtration may be entertained. With increase in plasma concentration the impedance would be reflected on a temporal basis in a progressive decrease in the percentage of drug filtered per unit time. It would follow that in some manner PAH either accentuates the attraction between plasma protein and the drug or decreases the selective permeability of the glomerular membrane. Preliminary experiments in this laboratory support this interpretation. On this basis the calculation of a 'corrected' clearance ratio based on the *in vitro* ultrafiltration of the drug from plasma through an artificial membrane has little or no interpretative significance.

Let us examine the conditions as they might be thought to obtain if tubular secretion of carinamide occurred and if only 'unbound' drug were filtered at the glomeruli. Under these conditions the amount of drug excreted per minute (UV) = (the amount secreted by the tubules + the amount filtered) - (the amount reabsorbed by the tubules). If there were no secretion or reabsorption, or if the two opposing mechanisms were equal functionally it follows that the clearance of carinamide would equal that of creatinine, i.e. the excretion ratio of carinamide to creatinine would equal 1.0. In order for the excretion ratio to be greater than 1.0 the amount secreted per minute at low plasma concentrations must be greater than the amount reabsorbed. Where the excretion ratio is less than 1.0 the amount secreted

would have to be less than that reabsorbed by the tubules, in order to have the interpretation compatible with the experimental data. From a functional standpoint this would appear to mean that the reabsorptive capacity for carinamide was much greater than the tubular secretory capacity. In order for PAH to decrease the amount of carinamide excreted per minute it (PAH) would have to depress selectively the tubular secretion of the drug. From an anatomical standpoint one would have to place the secretory and reabsorptive mechanisms in the nephron in such a position with respect to each other as to permit them to fulfill functionally the conditions defined by the data. None of the speculations as to how this could be done seems attractive at present.

In our present opinion the data are more compatible with the filtration impedance theory as it has been broadly conceived. However, both interpretations probably should be considered only as tentative hypotheses for the basis of future research.

SUMMARY

The experiments presented herein indicated that considerable amounts of carinamide and its metabolite(s) were adsorbed on plasma protein. They were adsorbed to the same extent and the adsorption curves corresponded to the typical adsorption isotherm. Desorption of the compounds from plasma protein readily occurred.

A reciprocal relationship between the plasma concentration and the clearance ratio or excretion ratio for carinamide and *total* carinamide was found to exist. The clearance ratios for carinamide and *total* carinamide never exceeded 1.0 at any plasma concentration; however, their excretion ratios ('corrected' clearance ratios) fluctuated widely from greater than 2.0 to less than 0.50, although they were the same for the two forms of the drug at similar plasma concentrations. PAH at high plasma concentrations markedly reduced both ratios regardless of whether the excretion ratio was greater or less than 1.0.

From the above observations it seems certain that a large portion of the drug filtered at the glomeruli is reabsorbed by the renal tubules. However, it is evident that at low plasma concentrations glomerular filtration of the drug calculated as 'unbound' in plasma water is inadequate to account for the amount of drug excreted per unit time. It has been proposed that plasma binding, or the electrostatic attraction between plasma protein and carinamide, impedes the amount of drug filtered on a temporal basis but does not limit filtration to the amount determined in a static *in vitro* system as unbound. Alternatively, it may be that both tubular secretion and reabsorption of the drug occurs. The inhibition by PAH may be reconciled with either alternative hypothesis, although insufficient evidence to present or accept either interpretation as unequivocal is available.

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CENTRAL CONNECTIONS FOR AFFERENT FIBERS FROM THE KNEE JOINT OF THE CAT¹

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NERVES supplying the knee joint of the cat contain myelinated fibers, some of which are as large as 16 or 17 microns in diameter (1). The fiber spectrum resembles that found in cutaneous nerves. Most, if not all, of the myelinated fibers are afferent in nature and enter the spinal cord over 3 and sometimes 4 dorsal roots (2). Their subsequent connections have not until now been studied experimentally and this report presents data concerning pathways in the spinal cord for impulses initiated in articular fibers.

METHODS

Cats were used in all the experiments. Two were decerebrated under ether anesthesia, while 15 were anesthetized with sodium pentobarbital given intravenously or intraperitoneally. The articular branches of the tibial and saphenous nerves, designated respectively as posterior and medial branches, were used. Two types of experimental procedures were carried out: 1) Articular nerves were stimulated and potential changes recorded from the dorsum of the spinal cord. In several experiments, attempts were also made to record from lateral funiculi. 2) Dorsal funiculi were stimulated and antidromically conducted impulses recorded from articular nerves.

The spinal cord and hind-limb structures were covered with warm mineral oil. Silver wire was used for stimulating and recording electrodes. Potentials were amplified, led to a cathode ray oscillograph and photographic records were usually taken.

RESULTS

Stimulation of Articular Nerves. When the posterior nerve was stimulated with single shocks, potential changes of relatively long duration were recorded from the

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dorsum of the spinal cord over a distance of 30 to 40 millimeters (fig. 1). Most of the articular fibers enter the cord over the 6th and 7th lumbar dorsal roots, to a lesser extent over the 5th lumbar root, and occasionally over the 1st sacral root as well. The slow potentials were usually maximal at the 6th and 7th lumbar levels and exhibited well-defined negative and positive phases. Cranially and caudally the positive phase disappeared rapidly, and the negative phase, while detected over a greater extent, sometimes 6 or 7 segments, changed in amplitude and slope characteristics (fig. 1). These slow potentials, while little affected even by deep anesthesia, were quite sensitive to asphyxia.

Spike potentials preceding the negative phase were not seen, except when using an amplifier with high frequency response, and then not uniformly. When present, they were small and could be detected only at levels of entry. The fastest of them conducted at rates of 90 to 100 meters per second.

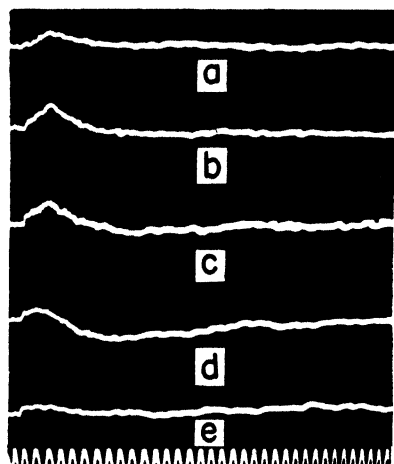


Fig. 1. SLOW POTENTIALS recorded from surface of ipsilateral dorsal funiculus after single volleys over posterior nerve. Electrodes were bright silver wire, one placed on the dorsum of the cord, the other thrust into adjacent musculature. *a*, Potential recorded at the 3rd lumbar segment; *b*, 4th lumbar segment; *c*, 5th lumbar segment; *d*, 6th lumbar segment; *e*, junction of 7th lumbar and 1st sacral segments. Distance between *a* and *e*, 40 millimeters. Time in 4 msec. intervals.

Most of the experiments utilized the posterior nerve. Comparable studies of the medial nerve in two experiments gave similar results, although the potentials were somewhat greater in magnitude.

All attempts to record spikes ascending in dorsal funiculi at more cranial levels were unsuccessful. The articular nerves are small so that relatively few funicular fibers are activated by stimulation of an articular nerve. Potentials are probably shunted, and increasing amplification merely tends to emphasize spontaneous activity. In a few experiments, attempts were made to record from the surface of the dorsal spinocerebellar tracts at upper lumbar and midthoracic levels, but these attempts were uniformly unsuccessful. Systematic exploration of these tracts with microelectrodes was not carried out, however, nor were recordings attempted in decerebrate preparations.

Stimulation of Dorsal Funiculi. With stimulation of dorsal funiculi, antidromically conducted impulses were readily recorded from articular nerves. Because of long conduction distances, there was considerable temporal dispersion (fig. 2). Only the initial deflections were used in the determination of conduction rates,

mainly because of possible complication by dorsal root reflexes (3). Section of ipsilateral dorsal funiculi below the level of stimulation abolished the responses, which could then be obtained by stimulation below the incision.

The maximum conduction rates observed at the levels of entry of articular fibers were 90 to 100 meters per second, but these rates decreased rapidly within the next few cranial segments so that at thoracic level the fastest rates observed were 40 to 60 meters per second. Still further decrease subsequently occurs, since at the first cervical segment the maximum rates were but 20 to 30 meters per second. The potentials recorded after stimulation at rostral levels were also smaller and much simpler in form (fig. 2).

Again, most of the experiments utilized the posterior nerve. In the two experiments involving the medial nerve, similar results were obtained and the maximum conduction rates observed were approximately the same.

DISCUSSION

Since Gasser and Graham's report (4), negative intermediary potentials of the type recorded in the present study have been attributed to internuncial activity. It may be assumed, therefore, that when articular fibers enter the spinal cord they give rise to a typical collateral formation by which synaptic connections are made, not only in immediately adjacent gray matter but also in that of more cranial and caudal segments. The longitudinal extent of gray matter activated by articular nerve fibers cannot be determined exactly, however, since internuncial activity at a particular level can probably be detected one or two segments away from that level. The segments to which articular fibers give collaterals may therefore be fewer in number than the 6 or 7 from which cord potentials could be recorded. Evidence of discharge over ventral roots is needed to localize the levels of synaptic connections which can be activated by single volleys.

Results obtained by stimulation of dorsal funiculi show that some articular fibers ascend in ipsilateral dorsal funiculi as far as the medulla oblongata. The rates of conduction over such fibers indicate that this ascending path is formed by processes of the larger myelinated fibers in articular nerves. Previous studies have shown that such fibers arise mainly from Ruffini-type endings in certain regions of the joint

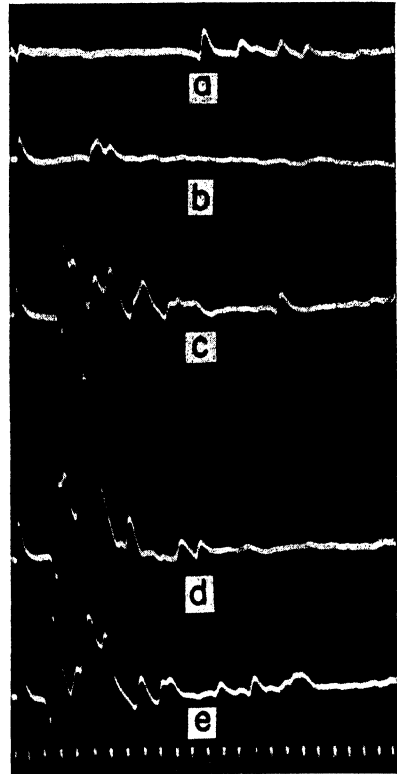


Fig. 2. ANTIDROMICALLY CONDUCTED IMPULSES recorded from the posterior nerve after stimulation of ipsilateral dorsal funiculus with bipolar silver electrodes. *a*, Stimulation of 1st cervical segment, conduction distance 489 mm.; *b*, thoracic cord, 319 mm.; *c*, 3rd lumbar segment 241 mm.; *d*, caudal part of 4th lumbar segment, 221 mm.; *e*, junction of 7th lumbar and 1st sacral segments, 181 mm. Time in msec.

and that most of them are 7 to 10 microns in diameter (1). A few are large enough to be classified as *Group I* fibers, but whether they are comparable in their anatomical connections to *Group I* fibers in muscle nerves could not be determined in this study.

The rapid decrease in conduction rates immediately rostral to levels of entry undoubtedly results from a decrease in diameter of the ascending fibers as collaterals are given off. Since a further decrease in conduction rate occurs in the cervical region, it is possible that collaterals are also given off here. The ascending fibers presumably relay in the medulla oblongata, but no studies of this feature have as yet been made. If articular nerves and their connections contribute in any way to position sense, impulses may well reach the cerebral cortex by this pathway.

Little is known concerning other ascending pathways. No positive evidence of conduction over the spinocerebellar tracts was obtained. Indirect evidence from previous experiments indicates that some articular fibers are concerned with pain mechanisms. Changes in respiration, pulse and blood pressure following stimulation of articular nerves resulted when such stimulation was strong enough to activate the smaller, more slowly conducting fibers (5). While no direct evidence is available, rostral conduction, after transmission through gray matter, no doubt would be mainly over lateral spinothalamic tracts.

In most of the experiments reported here the type and depth of anesthesia was such that reflex muscular responses were minimal or absent. Reflex activation of skeletal muscle following stimulation of small articular nerves may be readily studied only in decerebrate or decapitate preparation, and results of such studies will be reported in a later publication.

SUMMARY

Connections in the spinal cord of afferent fibers from the knee joint of the cat were studied as follows: 1) Articular nerves were stimulated by single shocks and the resulting activity recorded from the spinal cord. 2) Dorsal funiculi were stimulated at various levels and antidromically conducted impulses recorded from articular nerves.

The results indicate that most, if not all, articular fibers synapse with internuncial neurons shortly after entering the spinal cord. The larger myelinated fibers, those arising from Ruffini-type endings, also continue rostrally in ipsilateral dorsal funiculi to the medulla oblongata. Maximum conduction rates at levels of entry were 90 to 100 meters per second, but only 20 to 30 meters per second at cervical levels. The decrease probably results from a decrease in diameter of the parent fibers as collaterals are given off. No evidence of conduction over the spinocerebellar tracts was obtained.

It is a pleasure to acknowledge the technical help of Mr. Darwood Hansen, who is responsible for the design, construction and operation of much of the experimental equipment.

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EFFECTS OF OXYGEN DEPRIVATION UPON THE COCHLEAR POTENTIALS¹

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THE basic problem of the effects of oxygen want upon bodily functions has received an ever-increasing amount of attention in recent years, to a large extent as a result of the continued development of aerial flight at high altitudes (1). A part of this problem, which is our concern here, is the behavior of the ear and, more particularly, the action of the cochlea as revealed in its electrical potentials.

Only a few studies have dealt with hearing during oxygen deprivation and their results have been conflicting. Aggazzotti in 1904 (1a) reported a dulling of threshold acuity to sounds as a result of a reduction of atmospheric pressure. Lewis (2) in 1918 and Bagby (3) in 1921, however, observed no changes when the oxygen intake was reduced to the point of psychological failure and collapse.

Gelhorn and Spiesman (4) in 1936 reported significant reductions of sensitivity from moderately severe deprivations. They determined the thresholds of hearing, mostly for a tone of 1024 cycles, while a subject was allowed to breathe air that had been diluted with nitrogen, and in different tests varied the dilutions and periods of exposure. In a typical test they found that a mixture containing 10 per cent of oxygen (which is the equivalent, in terms of the partial pressure of oxygen exerted upon the alveoli of the lungs, of an altitude of about 19,000 ft.) when breathed for 10 to 30 minutes reduced the acuity by a significant amount.²

McFarland (5) in 1937 used a 2-A audiometer to measure the acuity of 6 subjects at sea level and after an ascent of the Andes mountains to an elevation of 17,500 ft. (equivalent to an atmosphere at sea-level pressure containing 10.7 % of oxygen). He reported a reduction of sensitivity for all the octave tones from 64 to 8192 cycles, a reduction varying in amount from 0.8 db at 64 cycles to 10 db at 8192 cycles. However, these results are not to be taken at their face value, for they may be accounted for merely as instrumental changes. Rudmose and his associates (6) have shown that a telephone receiver when applied to the ear in a standard manner fails to maintain its normal output of sound pressure when there is a reduction in the density of the air. The changes that they observed were of the same order of magnitude as those in McFarland's experiment. This instrumental effect will account also for the earlier observations of Aggazzotti.

To the above evidence may be added the common reports of airplane pilots that no depreciations of hearing are evident to them even under conditions in which vision is seriously affected, and up to the point where a loss of consciousness puts an

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² The amount of reduction was stated in 'per cent of normal sensitivity,' which to us is unintelligible. The Western Electric 2-A audiometer, the instrument used in the tests, gives readings in discrete steps of 5 decibels relative to a standard ('normal') level. We should suppose that the authors meant 'decibels' rather than 'per cent' were it not that they reported steps smaller than 5.

end to observations. These indications are in harmony with all but Gelhorn and Spiesman's results in showing a high resistance of the auditory sense to anoxemia.

Another approach to this problem is the electrophysiological study of the ears of laboratory animals. Our method is to measure the electrical potentials generated in the cochlea during stimulation with sounds under normal conditions and in the course of oxygen deprivation. Let us first mention some preliminary observations that throw light on the problem.

Probably everyone who has made an extensive study of the cochlear potentials has found that when the ear is stimulated by a steady sound (of not too great an intensity) the potentials maintain a constant value over extended periods of time, and indeed as long as the physiological condition of the animal is unchanged. When for some reason, as perhaps a faulty use of anesthesia, the physiological condition deteriorates as shown by labored, infrequent respiration and light, irregular heart action the cochlear potentials become impaired. Their impairment appears only *in extremis*: when the physiological deterioration has proceeded to a serious stage; and we have come to recognize this cochlear sign as a warning of the impending loss of the animal.

A foregoing study (7) dealt in detail with the changes of cochlear potentials that result from the animal's death. These potentials suffer a serious impairment just before the heart stops, and thereafter continue to decline rapidly for several minutes. When they reach a value of perhaps 20 db below normal (depending somewhat upon the cause of death) they decline less rapidly, and so continue for a matter of hours until they are no longer discernible. No doubt a number of physiological conditions are involved during this course of changes, but it became evident that one of the important ones is oxygen starvation of the cells in which the potentials are generated. The present experiment clearly establishes this fact.

PROCEDURE

This investigation was carried out on 11 cats. The animals were first anesthetized with Dial (diallyl barbituric acid) injected intraperitoneally, and then were curarized (with Squibb's intocostrin, by intravenous injection) to the point where reflexes were absent and respiration ceased. They were then maintained for the duration of the experiment by artificial respiration. It is important to note that these animals were in a state of minimal activity: the only remaining muscular exertion was that of the heart. The metabolic processes had only to sustain this action and a bare subsistence level of general cellular activity.

The inlet on the respirator was either open to the air—the 'normal' condition—or was attached by rubber tubing to a gas bag containing any desired respiratory mixture. The bag contained dilutions of air with nitrogen in varying amounts, giving oxygen compositions between 4 and 0.5 per cent. A gasometer was used to make up the gas mixtures, and it was placed in the respiratory line to indicate the rate of flow, which was kept at 1.5 liters/min. The heart action was regularly checked by listening with a stethoscope.

The cochlear potentials were picked up with an electrode on the membrane of the round window and were measured with a selective voltmeter (the General Radio

Type 736-A wave analyzer). The usual procedure consisted first of systematic measurements on stimulation with each of 12 frequencies from 100 to 15,000 cycles to ascertain the general condition of the ear. Then a 1000-cycle tone was adjusted to the intensity necessary to give a standard response, usually of 100 μ v., and thereafter it was steadily maintained at this level. Under normal conditions, as has already been indicated, this 100 μ v. response will continue with only minor variations for many hours and perhaps indefinitely.

The gas bag with its contents of diluted air was then connected to the inlet of the respirator, and observations of cochlear potentials were made at frequent inter-

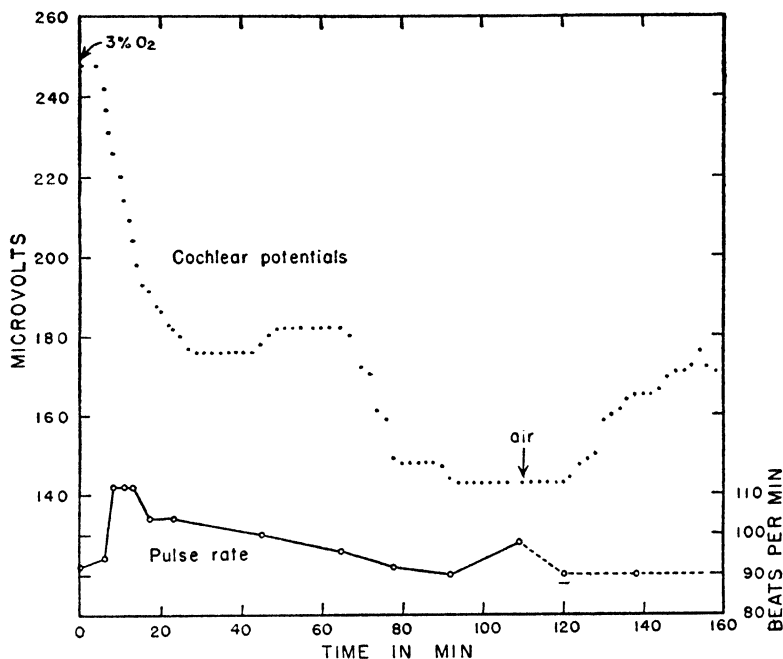


Fig. 1. EFFECTS ON COCHLEAR POTENTIALS of respiration with a mixture containing 3 per cent oxygen. The cochlear potentials are shown in microvolts by the series of points in the upper portion of the graph, and the pulse rate in beats /min. by the graph below. Arrows indicate when low-oxygen mixture was introduced and when air breathing was restored.

vals, usually every 15 sec. but sometimes oftener when changes were proceeding rapidly. Often the changes were followed to the point of death and somewhat beyond, but usually when the heart action became seriously impaired the gas bag was disconnected and air supplied to the respirator in the effort to resuscitate the animal. We have also used adrenalin, injected into the heart, and have succeeded sometimes in reviving an animal after the heart beat had become wholly imperceptible. After revival and a period of normal respiration, a further deprivation period ensued, and so on until the experiment was ended.

RESULTS

Our first observation was one already foreshadowed by the earlier discussion, that the cochlear potentials are notably resistant to anoxemia. Under the conditions

described, no changes were observed with gas mixtures containing more than 4 per cent of oxygen—mixtures equivalent to altitudes up to 40,000 ft. A little further dilution of the oxygen supply caused a slow, progressive deterioration of the potentials, until after an hour or two a level was reached from which there was little or no further decline. In such instances the animal evidently had made a physiological adjustment that sufficed, for a time at least, to maintain the cochlea without further deterioration at the level of function then reached.

A still further reduction of the oxygen supply caused a more marked effect, characterized by a rapid initial loss and then usually a leveling off. Figure 1 shows the results obtained on one animal with a gas mixture containing 3 per cent of oxygen. The cochlear potentials fell during the first 30 min. from an initial value of 248 μ v. to about 180 μ v. There they remained, with even a slight gain, for the next 40 min, reflecting a successful adjustment during this period. The adjustment could not be maintained longer, however, for the potentials then suffered a second rapid loss to a level around 145 μ v.

A study of the pulse record, shown in the lower curve of this figure, gives insight into the nature of the physiological adjustment. At the outset of the anoxemia the heart increased its rate of beating from 92 to 143 per min., which of course aided the interchange of oxygen between the blood and the bodily tissues. This high rate did not continue for long, but soon fell away, and after a time had sunk to a level too low to maintain the cochlear potentials at the 180 μ v. level. Then these potentials dropped to a lower level as shown.

After a period of oxygen deprivation of 110 min. the animal was respirationed with air, whereupon, after a brief latent period, the cochlear potentials rose steadily to a new level around 175 μ v.

Figure 2 represents an animal that was exposed to an oxygen mixture of only 0.5 per cent. The pulse rate was rapid to begin with, and first declined, then rose and finally fell precipitously. Around 30 min. the heart action was noticeably irregular, with short periods of no beating, and when at 40 min. its complete failure seemed imminent the respiration was changed to air. This measure failed and the heart stopped around 43 min. During this time the cochlear potentials fell continuously from an initial value of 100 μ v. to a value, at the moment the heart stopped, of 1.3 μ v.

Figure 3 represents an experiment in which the impairment of cochlear potentials was carried almost as far as in the preceding, and yet an effort to resuscitate the animal was successful. The respiratory mixture contained 0.75 per cent of oxygen, and in the first trial was given for 40 min. At the end of this time the heart action was weak and irregular, and the cochlear potentials had fallen from 105 to 6.5 μ v. Respiration with air strengthened the heart beat without changing its rate and raised the cochlear potentials to 27 μ v. A second trial with the 0.75 per cent oxygen mixture caused a rapid deterioration of heart action and reduced the cochlear potentials further to 1.2 μ v. The introduction of air at this point was too late, for though the heart action improved for a time it then weakened and ceased. The cochlear potentials showed a rise to 5.2 μ v. and then a fall. It is typical that this fall of potentials began a little while before the heart action showed any turn

for the worse, and that it underwent no particular change in its course at the moment of the heart failure.

It is possible, as figure 4 shows, to carry an animal through several periods of anoxemia, provided that each exposure is brief and the heart is not allowed to deteriorate too far. The animal represented here was exposed to a mixture containing 0.5 per cent of oxygen for three periods of about 10 min. each in quick succession and then after an hour of air breathing was given another period of exposure. Each period of oxygen deprivation caused a loss of cochlear potentials and each period on air gave a partial recovery of these potentials. The fourth exposure to the low-oxygen mixture caused the heart to fail badly and for a period of two minutes, just after air respiration was started, its beat could not be detected. Adrenalin was injected into the heart, and in two minutes more the pulse was strong and regular and the cochlear potentials had recovered markedly. A second injection of adrenalin was made to strengthen the heart further, and then this animal's ears were preserved for histological study by an intravital injection of fixing fluid.

It is clear from the results represented here that the cochlear potentials suffer from oxygen deprivation when this deprivation is extreme, and recover somewhat when adequate respiration is restored. Additional periods of deprivation cause further losses, and each again is followed by a partial recovery when air breathing is instituted. There is nearly always a net loss of cochlear potentials from each period of exposure, even though the animal may survive and judging from the character of its heart action may seem little the worse on account of its treatment. Yet the recovery of cochlear potentials is always limited, and a normal level is not regained even after protracted air breathing. As figure 4 shows, after the third exposure and the re-establishment of air breathing the level of potentials reached after 4 min. was not appreciably

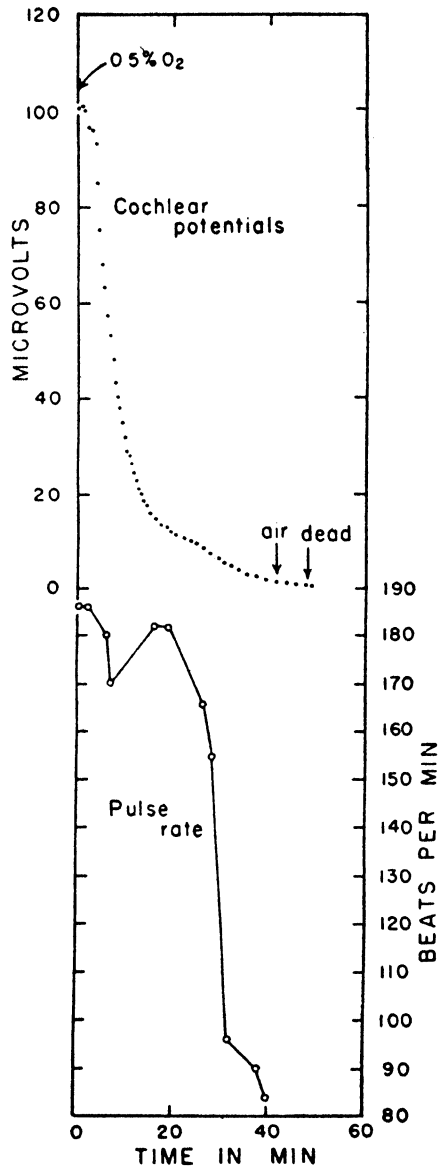


Fig. 2. EFFECTS of a respiratory mixture containing 0.5 per cent oxygen.

improved by as much as an hour's continuation of the air breathing. It is necessary to point out that this study has dealt only with acute conditions, and it remains to be discovered whether longer periods of time, or days or weeks, would restore the response further.

The results given so far were obtained with a single stimulus tone of 1000 cycles, and the question naturally arises whether all tones would present this same pattern of changes. McFarland considered this matter in his audiometric study, and believed that he had demonstrated a frequency relation—a greater susceptibility to loss in the higher range—but as we have seen his results are brought into question by the possibility of purely instrumental variations. The following experiment dealt with this aspect of the problem.

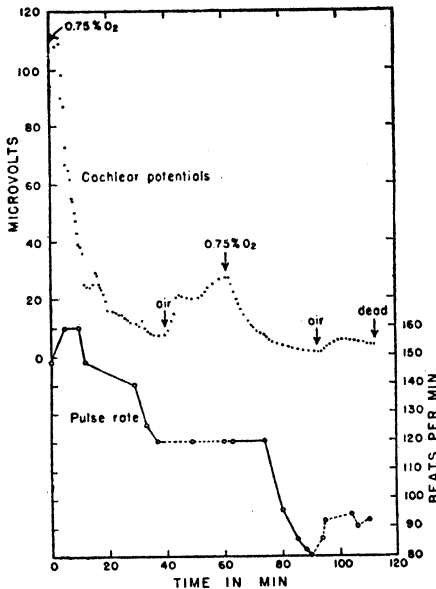


Fig. 3. EFFECTS of two exposures to a respiratory mixture containing 0.75 per cent oxygen.

again by presenting the various tones at the intensities necessary to produce the standard response. These measurements took 8 min. to carry out, and the results are of course subject to error on account of the progressive deterioration undergone during that time. We have sought to correct for this error by measuring the 1000-cycle response at the beginning and end of this testing period and adjusting the readings on the assumption that the deterioration thus shown was uniformly distributed over the period. The upright triangles in figure 5 represent the uncorrected readings and the inverted triangles the corrected readings. The corrected curve closely follows the form of the normal curve, with a mean difference of 42.6 db and a mean variation of 1.3 db. Hence it seems that the deterioration of cochlear potentials due to anoxemia is independent of frequency.

In one animal, represented in figure 5, a normal sensitivity curve was obtained before any anoxemia was introduced. This was done by ascertaining, for 12 different tones over the range from 100 to 15,000 cycles, the stimulus intensity necessary to produce a standard magnitude of cochlear potentials (a response of $1 \mu v$). The results are given in the lowermost curve of the figure, and show about the usual sensitivity function for the cat, with the keenest sensitivity for the middle and medium high tones. Then the animal was exposed to a mixture of 0.5 per cent of oxygen for 30 min., by which time the cochlear potentials had passed through the rapid phase of deterioration and were falling only slowly. The curve closely resembles that shown in figure 2, except that it leveled off sooner and at 30 min. was declining even more slowly. At this time a second set of sensitivity measurements was made,

DISCUSSION

As an examination of the first four figures will show, there were large variations in the temporal relations between changes in the oxygen supply and the resulting changes in the magnitude of cochlear potentials. When a respiratory switch was made the cochlear potentials sometimes began to show an effect after only a brief interval of 30 to 90 sec., and at other times this latency was as great as 8 min. The variations largely reflected the circulatory conditions then prevailing: the rate and

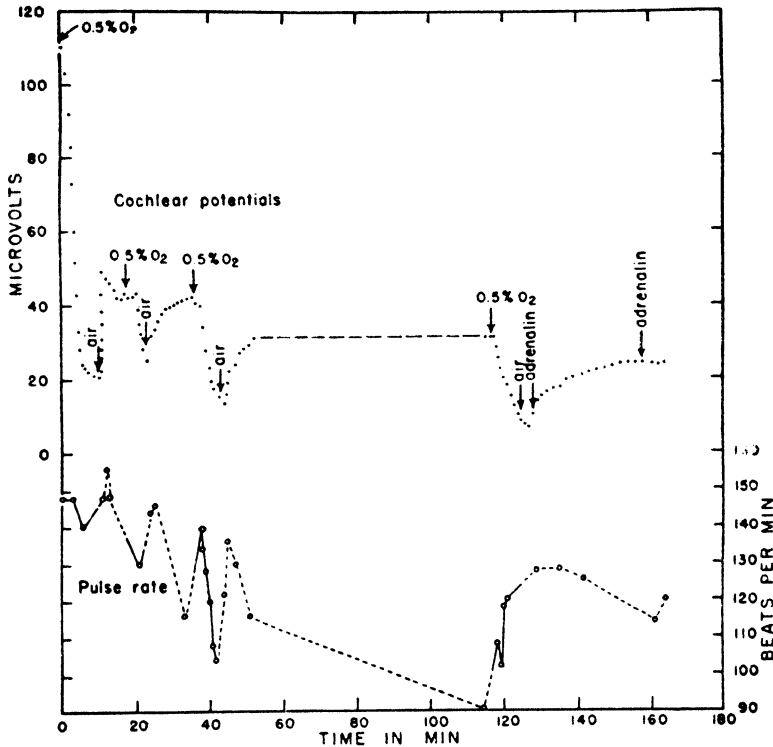


Fig. 4. EFFECTS OF REPEATED EXPOSURES to a mixture containing 0.5 per cent of oxygen. Near the end of the experiment, adrenalin was administered to facilitate heart action.

strength of the pulse, as we could observe, and no doubt also the dilatation of the capillaries and the degree of utilization of oxygen by the cochlear cells.

It is necessary at this point to consider the specific seat of the potential changes resulting from anoxemia. There is no doubt that these changes take place in the hair cells of the organ of Corti. Many lines of evidence—especially from examination of albinotic and other animals with malformed or atrophic cochlear structures and from experiments on stimulation deafness—point to these cells as responsible for the generation of the cochlear potentials.

If this site of the anoxic changes is the correct one, we are presented with a peculiar problem in view of the mode of nourishment of the hair cells. As is well known, these cells, along with the entire organ of Corti, do not possess any direct

blood supply. They evidently derive their nourishment from the endolymph, which, as Corti first suggested, is probably supplied by the stria vascularis, which is a band of pigmented cells lining the outer wall of the cochlear duct and richly served with blood vessels. The mean distance of this band from the outer hair cells (specifically, the distance from the middle of the stria to the middle row of hair cells) was measured in one cat and found to vary from about 0.20 mm. at the apical end of the cochlea to about 0.53 mm. at the basal end, and throughout the middle portion of the cochlea had a fairly consistent value of 0.33 mm.

Let us consider the structural relations in further detail. The hair cells are directly exposed to the endolymph only at their outer, ciliated ends. In addition, the

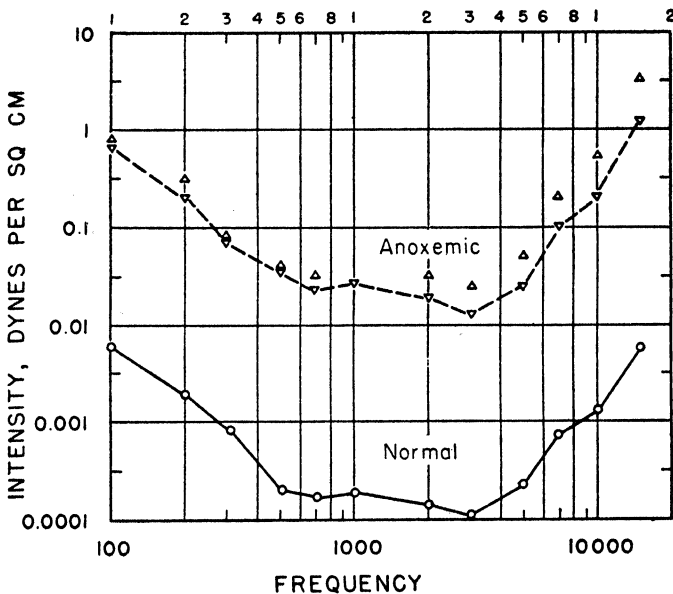


Fig. 5. ANOXEMIC EFFECTS as a function of stimulus frequency. Curves show the stimulus intensity required at various frequencies to produce a standard response of 1 microvolt, under normal conditions and in severe anoxemia.

outer hair cells have their midportions suspended in the fluid of Nuel's space, but this fluid, though common to the inner and outer tunnel spaces, does not communicate freely with the endolymph. The inner hair cells are closely surrounded by the phalangeal cells and the border cells. Hence it appears that oxygen transported by the red blood cells diffuses from the stria vascularis over a mean path of about 0.33 mm. across the cochlear duct, and then enters the hair cells directly through their ends or more circuitously through their side walls after penetrating the reticular membrane and the further intervening fluid or cells.

We need to judge as to the reasonableness of this conception of the nourishment of the hair cells in view of the latencies of cochlear potential changes observed in this experiment. Our observations are not ideal for this purpose, for we might better relate the potential changes to the oxygen content of the blood rather than to the respiratory supply. Still, these results provide the basis for a preliminary view.

Our minimal latencies, as stated, were around 30 sec., but these times include

certain external conditions. We must allow about 13 sec. for the gas entering the respirator to pass along the length of tubing leading to the animal, and some further time, which is difficult to estimate, for the replacement of the alveolar air. Then there is the time of the circulatory movement to the cochlea. These peripheral conditions will consume more than half of the observed time, and will leave something of the order of 10 to 12 sec. for the diffusion in the cochlea. This amount of time appears sufficient for the process. Yet there is no great excess of time, and we have to conclude that the hair cells do not have at their command any appreciable reserve of oxygen but must be furnished with a continual supply. Their needs though small are steadily demanding.

A comparison of the present results with those obtained earlier in the study of the death function reveals close similarities in the amount and temporal course of the cochlear potential changes, and indicates that the principal effect of death on these potentials is the stopping of the oxygen supply. We therefore feel warranted in carrying over to the present problem a principle established in the death study and not examined further here, namely, that the rate of deterioration of the cochlear potentials under these adverse conditions is unaffected by the intensity of stimulation (provided of course that this intensity is held below the level of physical injury). We have limited evidence here for still another relation found in the death study, that despite the deterioration of the responses the form of their intensity function is unaltered: their magnitude maintains its linear relation to the sound pressure at low and intermediate levels of stimulation and its distorted relation at high levels. Hence we conclude now, as we did in contemplation of the nature of cochlear activity after death, that the cellular processes concerned in the generation of the cochlear potentials are essentially simple in nature, and consist of a transformation of the mechanical energy of the stimulus into electrical energy and do not involve any liberation of stored energy.

We conceive that certain processes of a metabolic nature, dependent upon a continual supply of oxygen, provide a basis for this transformation but do not enter into it directly. Probably these processes have the duty of maintaining a base value of electric polarization in the hair cells. This polarization may be thought of as a surface positivity of the cell membrane. It is likely that under normal conditions, in the absence of stimulation, it is held at a fairly constant level and its average value still remains unchanged for ordinary stimulation. However, under the influence of sounds its instantaneous value changes, or at least its external influence changes, so that in the neighborhood of the cells there are electric fluctuations that are a faithful copy of the mechanical pressures. These fluctuations are the cochlear potentials.

Now when the oxygen supply is drastically curtailed the base polarization becomes reduced and in many cells is wiped out altogether. The sensitivity is therefore impaired. The cells whose polarization is lost of course no longer make their usual contributions to the electrical responses. The cells in which some polarization is still present will deliver their usual output as long as the stimulus is weak and the fluctuations do not exceed the polarization potential, but when the stimulus intensity is raised and the fluctuations are greater the output is impaired. Hence the response as a whole maintains its linearity only at the lowest response level and quickly grows distorted as this level is raised.

When the period of anoxemia is ended and normal respiration is restored the base polarization returns in some of the cells but remains absent or well below normal in many others. Therefore we find only a partial recovery of sensitivity.

We have made a histological study of the two ears of a cat that was exposed to extreme anoxemia over a period of 4 hours, and which during this time underwent a particularly severe loss of cochlear potentials. The animal survived until it was injected for the histological treatment. Microscopical examination of sections of the ear disclosed marked pathological changes in the organ of Corti over the whole length of the cochlea. The sensory structure had largely lost its differentiated character and was reduced to a misshapen mass in which cell nuclei were present but no cell boundaries could be made out. The arch of Corti could be seen, though it was greatly modified in form. No hair cells could be surely identified, though certain nuclei were present that may have belonged to the inner hair cells. On the other hand, the internal sulcus cells and the stria vascularis remained in fairly good condition. Reissner's membrane was greatly ballooned out and through most of the cochlea was closely adherent to the wall of the scala vestibuli. These changes fully account for the observed impairment of electrical responses.

SUMMARY AND CONCLUSIONS

Measurements of the electrical potentials of the cochlea carried out in cats have revealed deleterious effects upon the ear as a result of oxygen deprivation. The deprivation has to be severe and is produced by respirating with air containing less than 4 per cent of oxygen—a mixture that gives the same partial pressure of oxygen as that of the atmosphere at an altitude exceeding 40,000 ft. above sea level. As is well known, this degree of oxygen lack cannot be endured by man without loss of consciousness and even of life itself; and it was possible for the cats only because they were maintained at minimal activity under anesthesia and curare and were artificially respired.

As the anoxemia develops over several minutes the cochlear potentials undergo a rapid initial loss and then tend to level off. With extreme deprivations, which carry the animal close to the point of death, the losses may amount to 40 db and more. On a return to normal air breathing the responses recover, but only partially. Repeated exposures to anoxemia give a cumulative deterioration of the responses. The effects are found for all tones, without appreciable variation with frequency.

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SITE OF ACTION IN THE CENTRAL NERVOUS SYSTEM OF A BACTERIAL PYROGEN

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NUMEROUS investigators have confirmed and extended the observation of Isenschmid and Schnitzler (1) that the hypothalamus is essential for regulation of body temperature. Notable work in this field was that of Ranson and his co-workers (2) who clearly separated the heat loss center from the heat conservation center in the hypothalamus. It followed logically that fever result from disturbances of the hypothalamic thermoregulatory mechanism. Ranson, Clark and Magoun (3) tested this hypothesis experimentally but were unable to draw definite conclusions regarding the rôle of the hypothalamus in pyrogenic fever. The hypothesis was further weakened by results of Haertig and Masserman (4) who found that cats with massive hypothalamic lesions and impaired thermoregulatory ability developed fevers of infectious origin.

Recently a purified and relatively non-toxic pyrogenic extract of a *Pseudomoma* species that gives dependable febrile responses (5) was made available as a tool to re-investigate this problem. Efforts were made first to determine whether the pyrogenic response is mediated through the nervous system and, having established this point, to locate the level of neural integration of the pyrogenic response within the neuraxis.

MATERIAL AND METHODS

Dogs and cats were used. The pyrogenic extract, known as Pyromen (5), was usually employed but in several instances other pyrogens were tried. Dosages ranged from 20 to 200 $\mu\text{g}/\text{kg}$. of body weight. Intraperitoneal and intravenous routes of administration were employed in the cat; the intravenous route was used in the dog. Control runs were made with intravenous injections of pyrogen-free saline solution in many of the experiments. Rectal temperature was recorded continuously with a resistance thermometer on the Leeds and Northrop Micromax potentiometer in most animals. In other animals periodic rectal temperatures were determined with a clinical thermometer. In some experiments cutaneous and rectal temperatures were obtained with iron-constantin thermocouples and a potentiometer. Wherever temperature regulation was impaired as a consequence of neurological lesions, environmental temperature and humidity were controlled in a specially constructed room. In each experiment an attempt was made to obtain a balanced control period prior to injection of the pyrogen. In the absence of febrile responses animals were reinjected one or more times.

White blood cell counts were made in a few experiments. In several trials sodium salicylate and pentobarbital were employed in an attempt to break the fever. Atropine was used to prevent the gastrointestinal side reactions in one spinal dog. Vascular changes occurring in the rabbit's ear were studied in cooperation with Dr. R. G. Williams by means of the preformed tissue chamber.

Neurological lesions were made under aseptic conditions and with ether or pentobarbital anesthesia. Transections of the exposed spinal cord were performed with a sharp scalpel at various levels. Decortication was performed with a scoop. Massive bilateral thalamic and caudal hypothalamic

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lesions were placed in 5 cats with the Horsley-Clark stereotaxic instrument.² Decerebration with no attempt at asepsis was performed in 2 cats by the Sherrington scoop method, in 9 cats by the anemic method of Pollock and Davis (6) and in 8 cats by the Schmidt method (7). Chronic as well as acute preparations were studied, except in the case of the decerebrated animals which were studied during the first 48 hours following surgery. All neurological lesions were confirmed at autopsy. Spinal cord transections were verified by silver-stained serial sections through the region of the lesion. The extent of thalamic and hypothalamic lesions was determined on serial sections stained by the Weil technique. The level of anemic decerebration was established by gross inspection of the brain for the distribution of dye following injection of an aqueous solution of methylene blue under high pressure into the aorta.

RESULTS

Normal Animals. One hundred thirty trials were carried out on 23 cats. The response to administration of pyrogenic substances, while variable and occasionally absent, consisted typically of the following sequence of events: drowsiness, rising rectal temperature and falling skin temperature of the ears, nose and footpads during

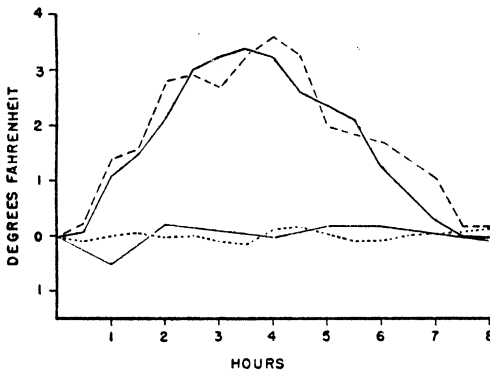


Fig. 1. CONTINUOUS RECORDS of rectal temperatures. The solid heavy line represents a typical response to an injection of Pyromen in an intact cat; the solid thin line represents the response to an injection of pyrogen-free saline solution in an intact cat; the dotted line represents the response to an injection of Pyromen in a cat 30 days after transection of the lower cervical spinal cord; the broken line is the febrile curve of a cat on the ninth day following massive bilateral caudal hypothalamic destruction. Injection was given at zero on the abscissa scale after the animals had been stabilized with respect to their rectal temperature.

the first 15 minutes, piloerection and moderate shivering during the second half hour. By the end of the first hour the febrile response averaged 2° F., with drowsiness and piloerection continuing but shivering ceasing. The fastigium was attained by 4 hours and averaged 3.6° F. at which time piloerection ceased. Defervescence then set in with a return of the temperature curve to the baseline by 8 hours (fig. 1). Occasionally fever occurred in the absence of overt shivering and piloerection. Retching, vomiting, defecation and urination occurred in some animals during the second half hour. Respiratory changes, chiefly in the direction of a rate increase, frequently occurred during the early period. Animals failing to yield a fever were given additional injections of the pyrogenic substances until the febrile response was obtained.

The pyrogenic response in the dog was qualitatively and quantitatively similar to that of the cat with several exceptions. The dog invariably responded to pyrogenic drugs with a fever (80 trials in 30 animals). The side reactions of vomiting, defecation and urination usually occurred; piloerection was much less pronounced and respiratory changes were more constant than in the cat.

² We are indebted to Prof. H. W. Magoun, of Northwestern University, for preparation of these animals.

Spinal Cord Transection. The spinal cord of one dog was transected at T-5, another dog at T-9, and a cat at L-1. These 3 animals yielded typical pyrogenic responses a day or two postoperatively when Pyromen was administered.

Transections of the spinal cord were prepared at C-7 or C-8 in 3 cats, at T-1 and T-2 in 2 cats and at C-7 in a dog. The cats were studied at various times from 5 to 30 days postoperatively; the dog was studied for 60 days postoperatively. These animals showed no appreciable ability to maintain a normal body temperature and had to be kept in a warm room. Even though the environmental conditions were maintained constant, each of these animals showed a slow cyclical variation in rectal temperature with elevations as great as 3° F. (apparently related to food intake). Twenty-one injections of Pyromen³, two injections of a similarly purified typhoid extract³ and one injection of 35 mg. of pyrexin⁴ failed to elicit a single unequivocal febrile response. *Cat 21* was tested 4 times with Pyromen and typhoid extract over a 30-day period and *dog 5* was tested 8 times with all three pyrogenic materials over a postoperative period of 60 days. In spite of the failure of pyrogens to elicit a febrile response in these high spinal animals, the cats occasionally had the usual side reactions. Retching, vomiting, defecation, urination, drowsiness and respiratory changes occurred in the dog during each trial in which pyrogens alone were given, but the gastrointestinal and urinary vesical changes were blocked by atropine whenever this drug was administered prior to the pyrogen. Slight reductions in skin temperature occurred in the dog but these appeared to be part of the visceral component of the mass reflex; subsequent prevention of skin temperature changes by atropine, which also blocked defecation and hence the mass reflex, bore out this impression. At no time was shivering seen caudal to the level of the lesion.

A marked leucopenia affecting all white cell types occurred in the dog with the cervical spinal cord sectioned, even though there was no febrile response. Leucopenia followed by leucocytosis during both pyrogenic and physical fever has been reported by other investigators (8, 9). We have seen these changes in intact animals and in man. Studies employing the preformed tissue chamber in the rabbit ear revealed intense vascular spasm during the chill phase after systemic administration of Pyromen. It was possible to observe the absence of adhesion of leucocytes to the cutaneous blood vessel walls; therefore such a process is believed not to have been a factor in the leucopenia. The findings in the cervical spinal dog appear to dissociate the peripheral blood cell changes from the febrile response.

Decortication and Massive Thalamic Destruction. One decorticate cat had only the pyriform area of the cerebrum intact and the basal ganglia were completely destroyed. This animal responded to injections of Pyromen with the usual febrile response as early as one day postoperatively. Another cat, with massive bilateral thalamic lesions, was tested four times from the 4th to the 10th postoperative day and exhibited a fever on each occasion.

Massive Bilateral Caudal Hypothalamic Lesions. Four cats were studied. A febrile response was seen as early as one day postoperatively and 2 animals showed re-

³ Both supplied by Dr. N. M. Nessel of the Baxter Laboratories, Morton Grove, Ill.

⁴ Kindly provided by Dr. Vally Menkin of Temple University.

sponses as late as the 25th postoperative day when the experiments were terminated. Sixteen trials were made. There were 13 definite febrile responses and only 3 equivocal responses. In no instance was vomiting, urination and defecation seen and in only one trial was there a suggestion of shivering and piloerection (fig. 1). These animals were all relatively poikilothermic.

Decerebration. Nine cats were decerebrated by the Schmidt technique and tested with Pyromen immediately after operation. Decerebrate rigidity was marked but the animals tended to lose heat rapidly at an environmental temperature of 85° F. The dorsal transection level was through the superior colliculus and the ventral level varied. In the animals in which the ventral level traversed the rostral part of the midbrain (7 in all) no febrile response was elicited with a single injection of Pyromen and the rectal temperatures continued to drop. The usual side reactions were present

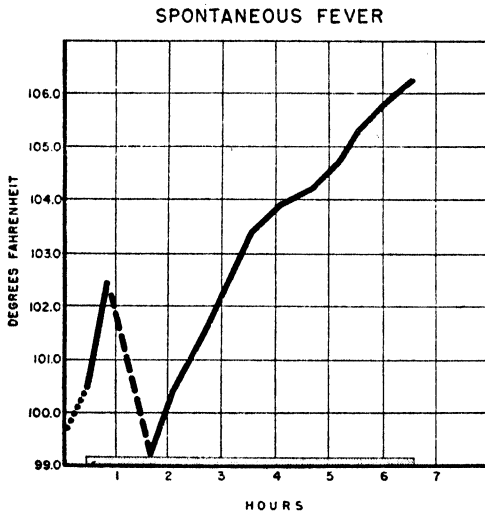


Fig. 2. CONTINUOUS RECORDING of the rectal temperature of anemically decerebrated cat 5 with a spontaneous fever. One hour after decerebration the rectal temperature was below 100° F. and radiant heat was applied (dotted line). Shivering set in (stippled bar) and the lamp was removed. When the rectal temperature had reached 102.5° F. the animal was placed in a refrigerator at 36° F. for 40 minutes (broken line). Upon removal from the refrigerator (solid line) the animal continued to shiver and the rectal temperature soared to 106.3° F., at which point respiratory failure occurred.

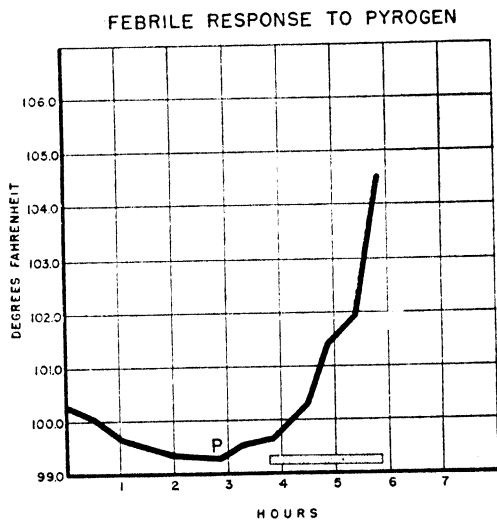
and severe respiratory embarrassment was encountered in all. This led to premature death in some instances as mucus plugged the tracheal canula and/or lung edema developed. In one cat with the transection passing just in front of the mammillary bodies a marked febrile response occurred; violent shivering persisted until death 80 minutes after the injection of Pyromen and at that time the rectal temperature had risen to 107.8° F. In the 9th animal the level of the transection was at the rostral border of the pons. This animal developed a spontaneous fever which was unaffected by a near surgical dose of pentobarbital. Prior to administering the pentobarbital, tonic and clonic convulsions were seen. The footpads were cool from the time of operation and throughout the experiment and shivering did not occur.

Neither of the 2 cats decerebrated by the scoop method and injected with Pyromen responded with a fever. They showed rapid declines in rectal temperature at an environmental temperature of 85° F.

Nine cats were decerebrated by the anemic method and studied immediately after surgery. The ventral level of ischemia varied from the rostral border of the

medulla oblongata to the rostral one-third of the pons. These preparations showed marked decerebrate rigidity and remarkable ability to maintain their rectal temperature at environmental temperatures as low as 76° F. Two developed spontaneous fevers (fig. 2). Four responded with fevers upon injection of Pyromen (figs. 3 and 4). Three gave only questionable pyrogenic responses. The 2 animals developing spontaneous fevers began to shiver and displayed peripheral vasoconstriction soon after surgery. Both had hyperpyrexia at the time of death and autopsy revealed considerable hemorrhage into the brain stem in the region of the ligature on the basilar artery. The unequivocal pyrogenic responses were characterized by the usual latency and drop in skin temperature but the animals showed more marked shivering and more constant and violent side reactions than were seen in intact animals. These experiments, because of premature respiratory failure, were of too short duration to

Fig. 3. CONTINUOUS RECORDING of the rectal temperature of anemically decerebrated *cat 2*. The recording began 20 minutes after termination of surgery. The Pyromen solution was injected at P. The stippled bar indicates the presence of shivering.



permit evaluation of the defervescence stage of the pyrogenic response. Decerebrate *cat 24* showed stepwise increases in rectal temperature following each successive injection of Pyromen (fig. 4). In decerebrate *cat 2*, two intravenous injections of 0.5 gm. each of sodium salicylate failed to reduce the rectal temperature.

DISCUSSION

The fever that occurs in a pyrogenic response is the result of increased heat production and reduced heat loss. The former appears to be due chiefly to an increase in skeletal muscle metabolism. In many of our experiments this was apparent in overt shivering. In other experiments overt shivering was not observed but an imperceptible increase in muscle tone, as shown by Burton and Bronk (10), may have been present and could have increased heat production. It is probable that some epinephrine is secreted during the chill phase of the fever and its calorogenic effect may have contributed to increased heat production. An additional source of body heat is provided by the increased metabolism that results from an increase in body temperature.

Reduced heat loss is brought about chiefly by cutaneous vasoconstriction and piloerection, the latter occurring particularly in the cat. Cutaneous vasoconstriction produces a reduction in skin temperature (11-14) that results in an appreciable layer of surface insulation, augmented further by the dead air spaces formed among the erected hairs.

The effectors involved in increased heat production obtain their innervation from the ventral horn neurons of the spinal cord and the cranial motor neurons to striated muscle. The latter, however, comprise but a small proportion of the total motor units. The smooth muscle of blood vessels and hair follicles receive their nerve supply from postganglionic neurons present in the sympathetic chain ganglia and indirectly from the intermediolateral cell column in the thoracic and upper lumbar spinal cord. This latter region also supplies some nerve fibers directly to the adrenal medulla. The main sensory inflow as well as the main motor outflow involved in fever

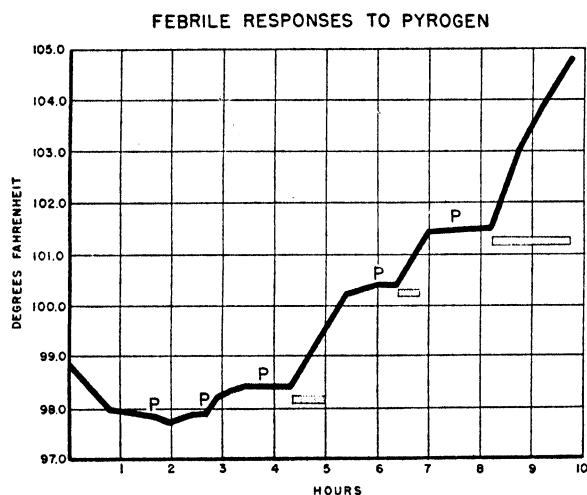


Fig. 4. CONTINUOUS RECORDING of the rectal temperature of anemically decerebrated cat 24. Injections of Pyrogen were given repeatedly at P. Shivering is indicated by the stippled bar.

is intact in an animal whose spinal cord is functionally separated from the brain stem at the lower cervical level. In view of consistent failure to obtain febrile responses from such preparations, even as late as two months postoperatively (a period far in excess of that necessary to insure recovery from spinal shock), it is concluded that the pyrogen acted neither on the spinal mechanism directly nor peripherally. This is further borne out by the absence of shivering caudal to the level of the lesion, the failure of the pyrogen to provoke piloerection and, finally, its failure to produce a decline in skin temperature when the mass reflex was abolished by atropine. The results obtained in animals with the spinal cord transected at midthoracic and lower levels, which gave febrile responses as early as two hours postoperatively, indicate that only part of the thoracic cord need be in functional continuity with higher nerve centers to produce fever. They strengthen the view that operative trauma and spinal shock did not seriously interfere with pyrogenic responses.

The remaining possibility, then, is that the pyrogen stimulated one or more coordinating centers in the brain and that these centers were required to be in functional

connection with the spinal and peripheral mechanisms. Decortication, massive thalamic and hypothalamic destruction and decerebration did not abolish the pyrogenic response. Since the lowest level of decerebration was at the rostral border of the medulla oblongata, at least the essential coordinating mechanism in the production of fever exists in the medulla oblongata and/or cervical spinal cord. Although the possibility of a center in the cervical spinal cord was not eliminated experimentally, one would suspect that the medulla oblongata contains the responsible integrating mechanism.

In so far as could be determined, destruction of most of the cerebral cortex, basal ganglia and thalamus did not alter any aspect of the pyrogenic response. The animals with massive hypothalamic damage and seriously impaired thermoregulatory ability, however, differed from intact animals; they did not shiver, piloerect, vomit or defecate (except in one experiment during which equivocal shivering and piloerection were noted). In the one Schmidt decerebrate preparation in which the caudal hypothalamus was intact a typical and exaggerated pyrogenic response took place.

The Schmidt and scoop decerebrate preparations with all or most of the midbrain intact never showed fever, shivering, piloerection or cooling of the skin although they did have the side reactions. In the large majority of animals with the level of decerebration at pontile or medullary levels pyrogenic or spontaneous fevers occurred. Shivering and cutaneous vasoconstriction were exaggerated and markedly protracted and the side reactions were consistently present and were severe. However piloerection appeared somewhat diminished. Another important difference was noted, namely, that at no time was a definite defervescence stage seen; the records often suggested that, had these animals lived long enough, extreme degrees of fever might well have occurred.

The pontile and medullary animals were prepared by the anemic method but the neurological signs and results of intra-aortic injection of dye under high pressure justify the assumption that the level of transection was as precise and as complete as if it had been made with a knife. The results of experiments in the one Schmidt preparation with complete anatomical transection extending from rostral border of the pons to the superior colliculus and with no midbrain tegmentum present, further support the conclusion that the pontile animal is able to have a coordinated febrile response. Furthermore, although the pontile animals were not specifically tested for their ability to thermoregulate, it was evident that they could maintain their rectal temperature in a cool environment more effectively than midbrain animals. These results suggest that there exists in the tegmentum of the midbrain a center that tends to inhibit shivering, vasoconstriction and the febrile response itself in the absence of the caudal hypothalamus.

In the experiments of Haertig and Masserman (4) infectious fevers occurred in cats that had massive hypothalamic lesions and were relatively poikilothermic. The lesions did not involve the caudal hypothalamus. Ranson, Clark and Magoun (3) prepared 21 cats with hypothalamic lesions and tested them with typhoid-paratyphoid vaccine. One of 6 cats with lesions in the caudolateral part of the hypothalamus responded with a good fever, and 6 showed marked falls in rectal temperature. Fifteen cats had more rostral hypothalamic lesions. Ten of these failed to show any

fever and the remaining 5 responded with only a very slight fever. Furthermore, their data demonstrated that there was a component in the vaccine which produced hypothermia. The pyrogenic material used in our experiments contains no factors that could be demonstrated to produce hypothermia.

SUMMARY

The response to injection of a purified pyrogenic extract of a *Pseudomonas* species was studied in cats and dogs, both intact and with central nervous system lesions.

Transection of the lower cervical and upper thoracic spinal cord in acute and chronic animals prevented the febrile response but not the side reactions. Cutaneous vasoconstriction and piloerection as well as shivering below the lesion did not occur. Transection of the spinal cord at or below T-5 did not prevent the development of fever. It was concluded that the febrile response to the pyrogen was mediated through the central nervous system and that the spinal cord needed to be in functional communication with one or more centers in the brain.

Decortication, thalamic and caudal hypothalamic lesions did not prevent the febrile response. Decerebrate preparations in which most of the midbrain remained intact failed to show a febrile response. However animals decerebrated at a pontile or medullary level had pyrogenic and spontaneous fevers. It appears that an integrating mechanism exists in the medulla oblongata and/or upper spinal cord which, when connected with the spinal cord, below the cervical region, was fully capable of evoking a febrile response to a pyrogen and in some instances of initiating 'spontaneous' fever.

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RELATION BETWEEN SPIKE HEIGHT AND POLARIZING CURRENT IN SINGLE MEDULLATED NERVE FIBERS¹

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THE effect of polarization on the height of the action potential (the magnitude of the 'alteration') in multifibered preparations has been the subject of many investigations, some dating back to the middle of the last century.² One of the most recent of these is included in Lorente's *A Study of Nerve Physiology* (2). In that monograph occur these statements: 1) "In the analysis of the problem" of electrotonic potentials "fiber diameter will eventually have to be taken into consideration;" and 2) "In the analysis of the problem that is possible at present the use of the single fiber approximation is justifiable." Some qualitative observations on the effect of polarization on the action potential of single medullated fibers were made in this laboratory in 1934 (3). The present investigation, an attempt to ascertain quantitatively the effect of polarization on the height of the spike of single fibers, was begun in 1943 and, after interruption by the war, was resumed in June 1947. A preliminary report of a part of the work was made in 1948 (4).

METHODS

The single fiber preparation has been mainly the sciatic nerve of the green frog together with the branch extending to the tip of the 3rd digit (the phalangeal or toe nerve preparation) (5). In about one out of three of these preparations there is a fiber of such outstanding excitability that it alone responds at the lead from the toe nerve to stimulation of the parent trunk with shocks that attain the threshold of that fiber. Twice only have we succeeded in obtaining comparable and adequate preparations by splitting the unbranched part of the sciatic nerve. The action potentials were recorded with the cathode ray oscillograph after passage through a resistance-capacity coupled amplifier. The networks used are shown in figure 1. Network *A* was the most usual one. Circuit *C* has certain disadvantages from the standpoint of the present investigation: when the anode lies between the stimulated locus and the lead it often blocks the fiber whose spike is being recorded, before the full range of the cathode effect has been achieved. This difficulty is minimized, however, by the fact that during cathode polarization the anode is on a thicker part of the nerve. An additional disadvantage is that it supplies conditions favorable to restimulation of the fiber beyond a block at the anode (6). The electrodes were of the calomel half-cell

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² A review of the early literature will be found in Schmitz and Schaefer (1).

type. The strength of the applied current is expressed in arbitrary potentiometric units and in many of the observations on single fibers the limiting strengths employed were those producing anode or cathode block or deformation of the spike. The linearity of the potential divider was tested by passing current through it in series with a dead sciatic nerve. The amplifier was checked for linearity of output.

The usual procedure has consisted of mounting the nerve vertically in the moist chamber (fig. 1) immediately after the dissection. If it seemed to contain a fiber of outstanding excitability it was then dripped slowly with Ringer's solution for some time while observing the spike elicited by threshold stimuli delivered at the rate of about one per second. Having thus obtained reasonable assurance that but a single fiber was responding, observations were begun. Often, however, the nerves were left in Ringer's solution for an hour or longer, some at room temperature, others at about 5° C., before beginning observations. Then, with the potential divider set commonly, though by no means always, at 40 on the linear scale of 100, the current source was adjusted to a strength that just sufficed to produce anode block. With this setting it

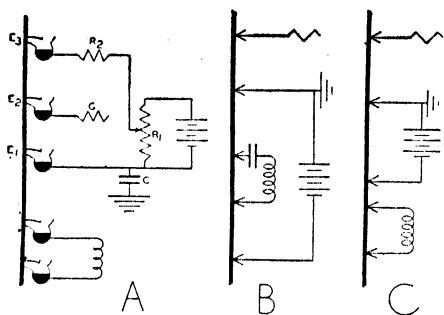


Fig. 1. NETWORKS. The resistance, R_2 , was 10 megohms with single fiber preparations and 2 megohms with sciatic nerves.

usually was possible to obtain current enough to attain cathode block within the range of the potential divider.

Since these tenuous preparations tend to dry it was necessary to carry through each set of observations as quickly as possible. For this reason the successive increments or decrements of current strength were made in steps of 10th of the range subtended by the potential divider. At each strength of applied current, both anodal and cathodal, the polarizing circuit was closed, and, after the attainment of amplifier equilibrium (a matter of a few seconds) two or three records were made of the conducted spike. Additional normal spikes were recorded at frequent intervals. A complete set of observations required about a half hour in the making. Often the set of observations was repeated after rewetting the nerve and chamber. With the high amplifications needed the noise level sometimes amounted to as much as 25 per cent of the height of the lowest spikes of a series—those recorded under strong cathode polarization. To minimize error due to this and to other sources it would have been desirable to have had several readings, not only two or three, at each polarizing current strength, but, as mentioned above, expedition was necessary.

In the great majority of cases the duration of the flow of the polarizing current beyond the time required for the establishment of amplifier equilibrium, a matter of about 3 seconds, was without effect on spike height. When, as occasionally has

happened, the height of the normal spikes, due presumably to drying, has increased progressively as the experiment has proceeded, the normal heights were plotted against the approximate times they were recorded and the normal height for each of the intermediate observations was obtained by interpolation. Observations were confined to the effects produced at the polarized locus. This was necessary because the toe nerve does not have an unbranched stretch that is adequate for comparative observations at different distances from the lead, but, more important, because of the risk of bringing spikes of other fibers into the picture. Sample records are shown in figure 2.

EXPERIMENTAL

Single Fiber Observations. Within ranges to be mentioned, the data on the relation between spike height and current strength with but few exceptions describe two

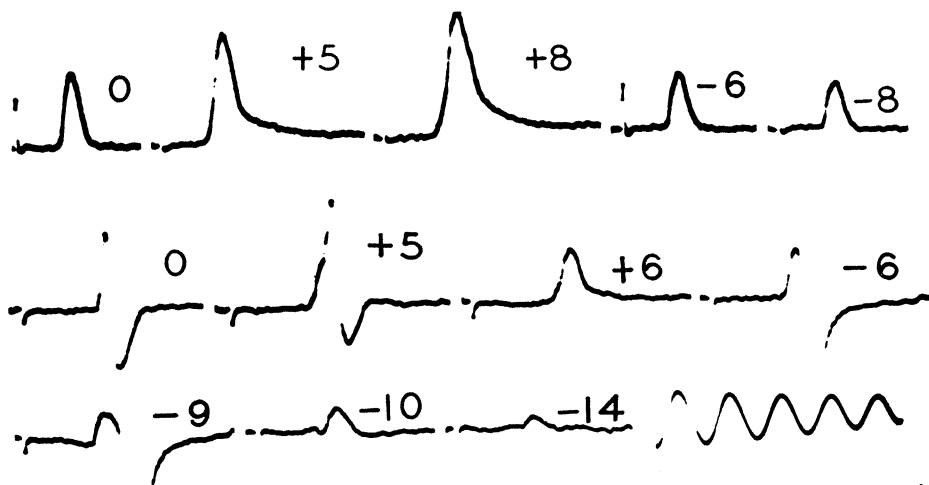


Fig. 2. A FEW OF THE SINGLE FIBER RESPONSES from two typical experiments. The numerals give the voltages in arbitrary potentiometric units. *Top row:* from experiment 6/17/48, monophasic recording. *2nd and 3rd rows:* from experiment 7/8/47, diphasic recording. 0, normal; +5, anodally polarized just short of block; +6, blocked anodally. -6, -9, -10, -14: increasing cathode polarization; -10, after block at notch near top of -9. Time, 1000 d.v./sec. Size as originally photographed.

straight lines meeting at zero at an angle that opens upwards (fig. 3 *B, C, D* and *E*); but in 3 of the 38 observations the graphs, again within the ranges to be mentioned, consisted of a continuous, inclined straight line passing through zero (fig. 3 *A*). If, within this range, there is any significant and regular departure from linearity on either side of zero it consists of a slight upward concavity on the anode side of the graphs. The eventual departure from linearity with further increase in current strength occurs, with but few exceptions, when the spike gives evidence of developing block. With anode polarization this almost invariably consists of the appearance of a notch somewhere on the up-stroke of the spike, as seen in figure 2, 2nd row, +5. At this time the rate of increase in height usually slows somewhat, but it may increase. More often, however, the linear increase continues quite to the point of block, when there occurs an abrupt and large fall in height (as from +5 to +6, fig. 2). No

attempt has been made to ascertain the exact configuration of the curve immediately preceding this event. The height attained by the spike at the time of definite deviation from linearity during anode polarization has, in this series, ranged between 118 and 182 per cent of the normal, with a mean of 151, an average deviation of 13.6 and an average deviation of the mean of 2.35. In the few cases in which the departure from linearity consisted of an upward bend of the curve the ultimate heights attained were greater even than the maximum just mentioned.

With cathode polarization the eventual departure from the linear decrease in spike height likewise may consist either of an upward (fig. 3 *D*) or a downward bend (fig. 3 *C*) of the curve. One of the factors that seems to determine the direction

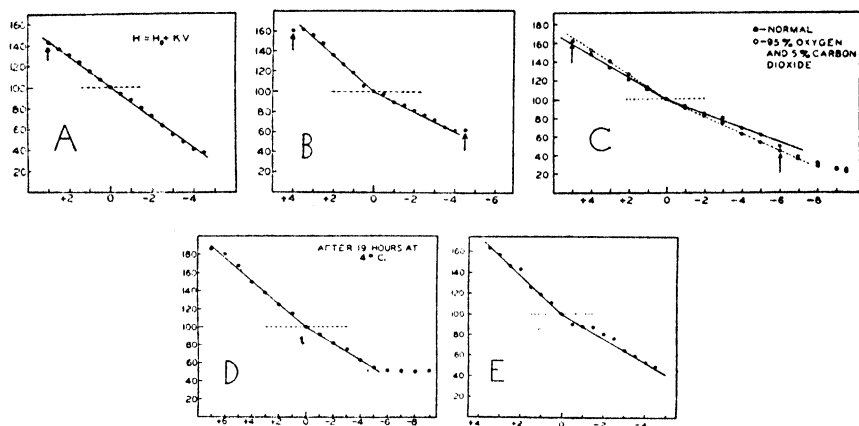


Fig. 3. GRAPHS OF SINGLE FIBER RESPONSES. *Ordinates*: spike height in percentage of normal. *Abscissae*: applied voltage in arbitrary potentiometric units. *A*, 6/12/48, normal. One of the three instances in which there was no rectification (tangent ratio of slopes = 1). Spike was notched at +3, block at +3.5; yet not blocked at -4.5. *B*, 6/17/48, normal. Spike was notched at +4, and at -4.5; not carried to block. Tangent ratio = 1.88. *C*, 5/22/48. Dots: nerve in air; tangent ratio = 1.47. Circles: in 95% O₂ and 5% CO₂; tangent ratio = 1.28. Notched at +5, block at +6; notched at -6, block (probably) at -8. *D*, 6/10/48. After 19 hours at 4° C. Spike notched at +6, block at +7; spike deformed at -6, but not yet blocked at -9. Tangent ratio = 1.44. *E*, 6/11/48. After 43 hr. at 5° C. Spike notched at +3.5, block at +4; not yet notched at -4.5. Considerable irregularity, but still an angle at zero. Tangent ratio = 1.59.

of the bend is the position on the spike at which the notch develops that presages the development of cathode block. If, as in -9, figure 2, the peak in front of the notch at which block will eventually occur is higher than the peak behind it, the curve of spike height is apt to bend upwards since the part of the fiber then determining the height of the spike is not so directly under the influence of the polarizing current; and if at the time of block the second peak is determining the spike height there will, of course, be a downward bend of the curve with the onset of block.

On the basis of some excitability determinations we have made it may be concluded that anode block occurs when the current attains the strength of 1 to 2 rheobases of the fiber in question, and cathode block at something less than 2 to 4 rheobases. Data supplied by 10 of the experiments show that the current strength required to produce cathode block averages 180 per cent of that producing anode block.

The current flow at the time of block is irrelevant, since it will vary with the size of the responding fiber relative to the cross-area of the nonresponding parts of the inter-electrode stretch. However, it has been of the order of tenths of microamperes.

In order to provide a means of comparing the results of the experiments in which the relevant data yield two straight lines meeting at zero strength, the ratios have been determined of the tangent of the angle formed with the horizontal by the linear portion of the curve expressing the results of anode polarization (in the 2nd quadrant) and of the angle (in the 4th quadrant) formed by the linear portion of the curve expressing the results of cathode polarization, each in relation to spike height. The data thus treated become independent of units of measurement. A ratio of one signifies that the current-strength spike-height curve is a continuous straight line passing through zero; values greater than one signify that the angle formed at zero opens upwards; while values less than zero would have signified that the angle opens downwards, but of the latter there have been no instances.

Thirty-four of the 38 observations on single fibers supply data relevant to this analysis. The tangent ratios derived from one and the same preparation under constant conditions are reasonably constant, but those from different preparations have ranged between 1 and 3.64. We have been unable to ascertain definitely the factors that determine these differences. In general, however, the lower ratios are found in experiments in which the recording was 'monophasic.' Thus in the 13 of such cases, the tangent ratios ranged from 1 (and included all three of the valid cases with this ratio) up to 2.41; but this, the highest, value was obtained from a preparation, which, though crushed at the distal lead, still yielded a diphasic record; and the next highest of these ratios, namely, 2.36, was obtained from a preparation that was monophasic, but not designedly so. The average of all of the ratios derived through monophasic recording was 1.53.

There are 28 instances in which the recording was diphasic, and in most of these the interlead distances were adequate for the complete recording of the up-stroke of the spike, as shown by calculation based on the duration of the up-stroke and the conduction velocity. In these the ratios ranged from 1.17 up to 3.64, and the average was 2.24.

Attempts were made to ascertain whether prolonged *storage of the toe nerves in Ringer's solution at 5° C.* affects their reaction to polarization. However, in only four of many trials did the preparation survive this treatment. In three of these cases, two after refrigeration for 19 hours (fig. 3 *D*), the other for 43 hours (fig. 3 *E*), the graphs were in every respect, quantitatively as well as qualitatively, like those derived from the fresh toe nerve preparations (fig. 3 *B, C*). The tangent ratios were 1.44, 1.59 and 1.81. The fourth preparation, refrigerated for 4 days, gave an anomalous result: each of three complete runs yielded curves that are best described as continuously concave upwards; the spikes attained a height of 160 per cent of the normal on the anode side before block and 73 per cent on the cathode side when the current had not yet attained blocking strength. There was no angle at zero.

Effect of CO₂. In three of the experiments observations were made first with the nerve in air and then, after the attainment of equilibrium, in an atmosphere consisting of 95 per cent O₂ and 5 per cent CO₂. In two of the instances the recording was di-

phasic; in the other, monophasic. In one of these experiments the spike was deformed, making accurate analysis impossible, but there was no reason for believing that the result in this case differed in any essential manner from the consistent results of the other two experiments. The tangent ratios in the two completely satisfactory observations were, respectively, 2.19 in air, 1.95 in CO_2 and 1.17 in air, 1.28 in CO_2 (fig. 3 C). In both cases the ratios are slightly smaller in the CO_2 treated nerves, but the differences are small and can hardly be regarded as significant.

That the CO_2 was exerting its characteristic effects is indicated by two, possibly three, of the reactions. 1) In all three of the cases the shock artifacts were larger during the exposure to CO_2 , and in two of the cases it was noted that the stimulating

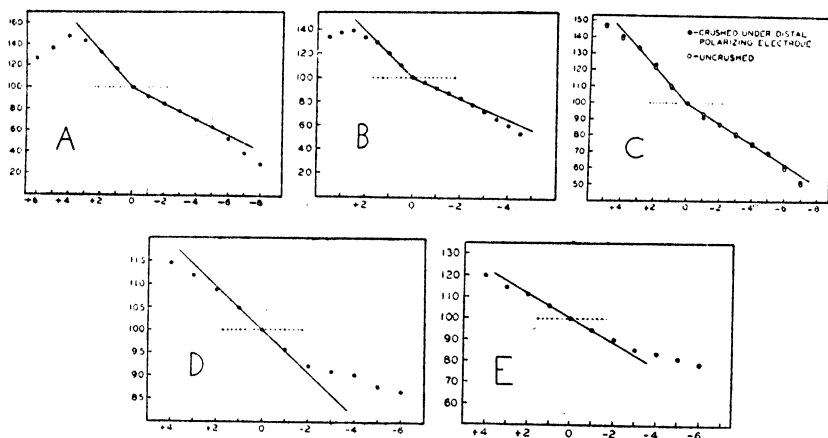


Fig. 4. GRAPHS OF SCIATIC NERVE EXPERIMENTS. *Ordinates*: spike height in percentage of normal. *Abscissae*: applied voltage in arbitrary potentiometric units. A, 5/11/49, A.M. Fresh bullfrog sciatic. Probably departing from linearity after height 118% and after height 62%. Tangent ratio of slopes = 2.14. B, 5/11/49, P.M. Bullfrog sciatic, mate of A, after storage 6½ hours in Ringer's solution at room temperature. Probably departing from linearity after height of 120% and after 78%. Tangent ratio = 2.2. C, 7/7/48. Fresh bullfrog sciatic. Probably departing from linearity after height 125% and at height 69%. Dots: after crushing under distal polarizing electrode. Circles: before crushing. The absolute voltage steps are considerably smaller in C, D and E than in A and B. Tangent ratio = 1.73. D, 7/10/48. Bullfrog sciatic after 2 days in Ringer's solution at 5° C. Inverted S in shape; no angle at O. E, 7/15/48. Bullfrog sciatic after 8 days in Ringer's solution at 5° C. Inverted S in shape; no angle at O.

current had to be increased, both observations indicating that the CO_2 had raised the fiber's threshold (7). 2) In all three of the experiments the CO_2 increased the susceptibility of the fiber to anode polarization as indicated by the strength of current required to block the impulses (2). However, the ratio of anode current to cathode current at blocking strengths in the CO_2 treated nerves was, as in normal nerve, approximately as 1 is to 2 (3). In all three of the experiments the curves derived from the CO_2 treated preparation show a slight clockwise rotation around zero (fig. 3 C). Possibly this is indicative of a change in the resistance of the nerve with a corresponding change in flow of polarizing current through it.

Alteration of other conditions, such as temperature or doubling the concentration of KCl in the Ringer's solution bathing the nerve (one experiment each), did not alter appreciably the form of the graphs or the tangent ratios.

Observations on Sciatic Nerve. For purposes of comparison, similar data have been obtained from the unbranched stretch of the sciatic nerve of the frog. The stimuli were 10 times half maximal for alpha. Since the primary aim was to ascertain whether graphs based on data derived from multifibered preparations also are linear on either side of 0, and, if so, through what range, the current strength, as a rule, was carried through a rather narrow range,—only twice to the point where there was an actual decrease in the height of the spike with anode polarization (fig. 4 *A, B*).

Relatively freshly prepared nerves were used in 10 of these experiments, 9 from the bullfrog and 1 from the green frog. Observations were begun usually within an hour after the dissection. In seven of the experiments the recording was diphasic, and the interlead distances (21 to 35 mm. in the bullfrog) were ample for the recording of the complete up-stroke of the spike. In the three experiments with monophasic recording the interlead distances were 10 mm. in two of the cases and 12 mm. in the other.

In the graphs of the data derived from the 7 preparations with diphasic recording and from the monophasic preparation with the 12 mm. spacing of the leads, the middle sections again consist of two straight lines emanating from zero at an angle (fig. 4 *A, B, C*). The tangent ratios of the slopes were 1.54, 1.70, 1.73, 1.76, 1.85, 2.14, 2.22 and 1.82 (the last from the green frog); there still is some variation in the width of the angle from preparation to preparation but the range is narrow in comparison with that obtaining in the case of the observations on single fibers. The two nerves yielding the tangent ratios of 2.14 and 2.22 were from the same bullfrog; the first (fig. 4 *A*) was used shortly after preparation, the second (fig. 4 *B*) after storage for 6½ hours in Ringer's solution at room temperature. Excepting a slight counterclockwise rotation of the graph derived from the stored nerve, which could be due to a difference in the potential subtended by the potential divider, the two graphs and the two tangent ratios are essentially alike.

In those instances in which the strength of the polarizing current was carried to the point where there was a change in the rate of change in height, the initial departure from the linear, as with single fibers, consisted sometimes of a slight upward bend of the curve, sometimes of a downward bend. With diphasic recording and anode polarization this bending of the curve occurred at approximate spike heights of 112, 114, 118, 120, 127 and 133 per cent of the normal. These values, obtaining at the time of changing inclination of curves, are roughly of the same order of magnitude as the lowest values found in the comparable observations on the responses of single fibers. In the two instances in which anode polarization was carried far enough to cause a marked decrease in spike height (fig. 4 *A, B*) this decrease began when the heights attained were 140 and 147 per cent of the normal. With cathode polarization the bending of the curve developed when the heights attained were approximately 62, 69, 74, 78, 82 and 84 per cent of the normal.

The curves derived through monophasic recording were not consistently alike. When the interlead distance was 12 mm. the curve mounted straight to a height of 148 per cent, and under cathode polarization it departed from linearity at a height of 64. However, the curves derived from the two experiments with the interlead distance of 10 mm. differ completely from the form that is usual, one of the graphs consisting of two inclined parallel, nearly straight, lines, the continuation of the 'anode' line lying

slightly above the continuation of the 'cathode' line, the other consisting of a curve slightly and continuously concave upwards.

Similar observations have been made on *bullfrog sciatic nerves after storage in Ringer's solution* at about 5° C. for periods ranging from 1 to 8 days. (A 12-day nerve was inexcitable, and, as mentioned above, a 6½-hour nerve behaved quite like its fresh mate.) In 8 of the 9 experiments the recording was diphasic with interlead distances ranging between 24 and 34 mm. Within the relatively narrow range of current strengths used in this series of experiments, the graphs derived through diphasic recording have, or approximate, the form of the middle section of an asymmetrical, reversed S: the curves (fig. 4 *D, E*) pass through zero with a gradually changing slope, the ordinates on the anode side usually increasing somewhat faster with increasing current strength than on the cathode side. The change in slope on either side of zero is so gradual that the middle sections of the curves can be regarded as essentially straight. In the one case with monophasic recording (a 3-day nerve with interlead distance of 8 mm.) the graph consisted of an inclined straight line passing through zero and bending upwards at both ends. Conduction velocities were of the same order of magnitude in the stored as in the fresh nerves.

DISCUSSION

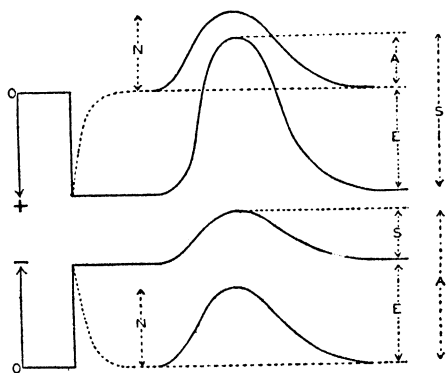
The first of our observations that requires consideration is the finding that the height of the conducted spike of a single fiber as recorded at the polarized locus, within certain limits, varies linearly with the strength of the polarizing current on either side of zero. In this connection it is necessary to recall how a resistance-capacity coupled amplifier performs under our experimental conditions. It records the spike but not the electrotonic potential that is established some 3 or more seconds earlier, since, in the interval, the condensers have filled and the base line consequently has returned to zero, where it is when the spike records. At the same time, however, the membrane potential, with certain qualifications (2), has been changed by the algebraic addition to it of the electrotonic potential; and when the membrane breaks down the height of the spike that results is the sum of the electrotonic potential and the potential of alteration or action, as illustrated in figure 5.

Since, in our experience, the relation between the height of the spike of a single fiber and the applied current, both positive and negative, is within certain limits linear and since, in our experience, the same relation usually obtains also in the case of the fresh sciatic nerve of the frog, though usually through narrower limits, it is logical to conclude that in these preparations *all* of the processes that are altered by the applied currents are altered linearly within those limits. This conclusion is inconsistent with the relevant results Lorente (2) has obtained in his polarization experiments on the sciatic nerve of the frog as seen in the two of his graphs (vol. II, figs. 1 and 2, pp. 36 and 37) illustrating the relation between the electrotonic potential (not spike height) and the strength of an applied current. Of these his figure 1 shows this relation as determined at the polarized locus with the multifibered preparation in air, and his figure 2 the result as obtained at a distance of 3.5 mm. from the polarizing electrode with the nerve in an atmosphere consisting of 95 per cent O₂ and 5 per cent CO₂. When these two curves of Lorente are plotted as we have plotted our data,

namely, the results of anode polarization in quadrant 2 and those of cathode polarization in quadrant 4, it becomes evident that the resulting curves have the form of a reversed S, are without an angle at zero, and consequently closely resemble in form the middle sections of the curves of spike height against polarizing current that we have obtained only, and practically without exception, from the bullfrog's sciatic nerve after storage in Ringer's solution at 5° C. for 24 hours or longer. The latter result, it might be added, serves as a check on our methods, since it shows that they are capable of exposing a non-linear reaction to polarization when it exists.

Lorente's graphs indicate that the deviation from linearity of the relation between strength of polarizing current and electrotonic potential is particularly marked in nerves exposed to an atmosphere containing CO₂. It was for this reason that we performed, with the single fiber preparation, the comparable experiment—the determination of the effect of CO₂ on the spike-height current-strength relationship. The addition of the CO₂ did not demonstrably alter the linear relationship from

Fig. 5. DIAGRAM ILLUSTRATING MODIFICATION of height of a spike as recorded by a condenser coupled amplifier when equilibrium has been established after the addition of a constant source of potential. *S*: recorded spike height. *A*: potential of alteration or action. *E*: electrotonic potential. Spike height during anode polarization = $A + (+E)$ and during cathode polarization = $A + (-E)$.



that found with the nerves in air. This observation should not be taken to signify that CO₂ never alters this relationship, since it is possible that with us the nerves prior to their exposure to CO₂ may have been in a state similar to that produced by CO₂. That, however, the treatment with CO₂ did change the state of the fibers characteristically is proved by an associated decrease in their excitability, by an increase in their relative susceptibility to anode polarization and by a clockwise rotation of the graphs around zero.

As has been said, with single fiber preparations this linear relation on either side of zero holds up to current strength that block the nerve impulse, or that begin to produce the deformations of the axon spike that presage block. Recently Rosenblueth *et al.* (8) have questioned the evidence upon which the conclusion was reached in this laboratory (3, 6) that the one or two successive 'quantal' changes in the spike of a single fiber that occur as the strength of a polarizing current is increased are due to block. They say that when, in their experience, they were certain that only one fiber was responding, in no case did they see sudden decreases in the spike, even when they polarized anodally or cathodally until total extinction of the response took place. Ignoring the conclusive portion of our published evidence proving that the 'quantal'

changes we have described are due to block in a single fiber, they assert that the pictures we have published as evidence of that are the result, not of block in one fiber, but of the play of the responses of two fibers.

As proof of this assertion they present two records (their fig. 1 *C, D*), both from the same root preparation, which they say "are quite similar to" the pictures which we have accepted as evidence of block in a fiber. We can agree with them that *C* and *D* are made up of the responses of two fibers; no one could possibly have regarded them as responses of but a single fiber. That *C* is made up of two fiber spikes is proved, as they point out, by the presence of tell-tale latency shifts of only the upper part of the record, indicating that the stimulus is just threshold for the fiber making that contribution to the picture, but well above the threshold of the fiber making the lower contribution. That *C* and *D* each is composed of the spikes of two fibers is indicated also by the position of the notch on the up-stroke—it is too far in front of the crest of the lower spike. In polarization block of a single fiber the part of the picture about to disappear rises, typically, out of the crest of what remains at block. Even in experiments in which the conduction distance is as long as 130 mm., as was the case, approximately, in all of our earlier experiments, the divergence from this relation was rarely greater than 0.2 msec. (3). The conduction distance in the case of their figures *C* and *D* could not, with their preparations, have been greater than 35 mm.; yet the upper spike of their figure rises out of the lower 0.25 msec. ahead of the crest.

In our experience, the really difficult double-fiber response to identify as such is when there are two fibers with identical excitabilities and conduction velocities. But even under such circumstances, it must be rare, indeed, that during the course of a prolonged experiment, involving the appearance on the screen of many hundred threshold responses, something does not transpire to disclose the compound nature of the response. Their "good test for the all-or-nothing character of the response" differs from the one we have used through the years and in the present research, only in respect to the frequency of stimulation; their threshold stimuli are at the rate of 50/sec., ours about once per sec., but through long periods of time.

Additional evidence (all of which is on record) on which we base the conclusion that the 'quantal' changes in the spike are the result of successive blocks along the course of a single fiber is, to consider now for the sake of brevity only the effects of anode polarization, as follows:

1) It is not unusual for the 'quantal' changes, when there are two of them, to reduce the height of the spike to $\frac{1}{2}$ or even to $\frac{1}{3}$ its initial value (3), and this residue, moreover, is anodally polarized. If such 'quantal' decreases were the expression of the elimination in succession of the spikes of different fibers, then, since there is in general a direct relation between spike height and conduction velocity (5), the conduction time should increase markedly with each deflection, but if it changes at all the change is in the direction of a slight decrease.

2) Needless to say, if the two or three sudden changes in the spike that occur as polarization is increased were pictures derived from as many fibers, the fibers would have very different diameters and consequently correspondingly different thresholds, but the threshold remains the same throughout such an experiment.

3) The information supplied by records obtained with diphasic recording is unmistakable, yet Rosenblueth *et al.* fail to mention this phase of our published evidence. When a single fiber is responding the invariable result, provided the increase in current strength is carried far enough, is that with the first 'quantal' change the record becomes completely monophasic—the impulse is completely blocked (cf. 'S₂' spikes of fig. 1, 6). If, with further increase in current strength a second and a third abrupt decrease in height occurs, the quantitative relationships are the same as with monophasic recording, and what we have said in that connection applies here. Now if, say, two sudden 'quantal' decreases in spike height were the expression of the participation of 3 fibers in the initial pictures, one would expect the largest fiber to have not only the fastest conduction velocity but also the greatest susceptibility to polarization (9). On this basis it should be the first to block as the current strength is increased, and at that stage the spikes of the still unblocked smaller fibers should remain diphasic and become monophasic only after further increases in the strength of the polarizing current; but, as a matter of fact, the change to monophasicity invariably is all or none when the response is that of a single fiber. There are, however, conditions under which a blocked impulse can re-initiate the impulse beyond the block (6); those conditions are of no present concern.

Rosenblueth *et al.* (8) say that as a means of securing the responses of a single fiber their spinal root preparation is a 'compromise' between giant fibers and our toe nerve preparations. This, they imply, is because their preparations are less than 70 μ in diameter and were found to contain 'several' functional nerve fibers. The toe nerve preparation at the point led from has a diameter of 20 to 50 μ and by actual count contains 20 to 100 fibers ranging down from a diameter of 12 μ (5). A root preparation 70 μ in diameter could contain more than 27 fibers measuring 10 μ in diameter. Under the circumstances, can it be assumed that the word 'several' implies that only a few of the constituent fibers of root preparations conducted from end to end? If so there can be but little difference between their root preparation and our toe nerve and split sciatic preparations with respect to a) the number of contained fibers or b) the possibility of local variations in diameter and associated local demarcation currents which might facilitate the development of local blocks under polarization.

Rosenblueth *et al.* argue, as does Lorente also (2), that the myelin sheath could not act as an insulator. In support of this position the former refer to the experiments of Tasaki (10) indicating that myelin is not a perfect dielectric, but not to the statement by Tasaki in the same paper that "Die internodale Strecke der Faser lässt sich nicht erregen," nor to a later publication by Tasaki *et al.* (11) in which it is stated that the action current of a nerve fiber derives from the Ranvier nodes and not from the myelin-covered region of the fiber. Moreover, complete support for our conclusion with regard to the location of blocking points at nodes is supplied by the experiments of Huxley and Stämpfli (12) who find that the membrane current through the myelin can be explained as a passive current through a resistance and capacity in parallel, and conclude "that the action potential at each node excites the next node by currents flowing forward in the axis cylinder and back in the fluid outside the myelin sheath." This result in some respects is similar in its implications to results

obtained by Blair (13). The latter found that "in depressed nonconducting medullated nerve, the all-or-nothing response of a single segment is the least obtainable; increasing the strength of stimulation increases the spike through the entrance of additional segment responses."³

Huxley and Stämpfli found that immersion of a nerve fiber in oil alters its reaction to isotonic sugar solutions; so, suspecting that the oil might affect also the distribution of current lines, they devised a method that made it unnecessary to bring oil into contact with the fiber. In attempting to account for the differences between Rosenblueth's and our results a similar thought had occurred to us, also. In any event, however, the behavioral differences of these two preparations, the phalangeal nerve and the spinal root, under polarization remain to be reconciled.

Within specified limits, the graphs derived from single fibers and from fresh, multifibered preparations are described, with but few exceptions, by the formula $h = h_0 \pm kv$, where h_0 is the height of the normal spike, v the polarizing voltage, the constant k , which certainly must be a function of the membrane resistance, being as a rule larger, but never smaller, when the polarizing electrode is the anode. The latter result is in keeping with the long known fact that the electrotonus produced by anode polarization is greater than that produced by cathode polarization, a difference that is usually regarded as evidence of the action of the membrane as a physical rectifier, but, by Lorente, as a reaction on the part of nerve.

To refer to some of the more recent of the relevant literature dealing with this subject, Cole and Hodgkin (14), using single giant nerve fibers of the squid and alternating current bridge methods, at first were unable to demonstrate 'rectification.' Then Cole (15) found that it could be demonstrated by killing the nerve under one of the polarizing electrodes and concluded that in their first set of observations the rectifying action had been concealed by approximately equal and opposite rectifications at the anode and at the cathode. Another reason, Cole believed, was failure in their earlier experiments to use polarizing currents of adequate strengths. Then Guttman and Cole (16) found that after killing the fiber at one of the polarizing electrodes the 'rectification' effect could be directly observed through the use of a direct current Wheatstone bridge. In the present investigation killing the locus of the toe nerve or of the fresh sciatic nerve over the distal polarizing electrode (with arrangement A, fig. 1, and diphasic recording) has not altered consistently the width of the angle at zero, although, owing to the proximity of the polarizing electrode to the distal lead, the diphasicity often was reduced somewhat thereby in the case of the toe nerve. In this connection it may be mentioned that according to Hodgkin (17) the membrane of the giant fiber of *Carcinus* obeys Ohm's law over a wide range of anodic current but shows marked deviations with cathodic currents. In the latter respect his result differs from that found here with frog's nerve.

The wide range, in different single fiber preparations, of the width of the angle formed at zero in the graphs of the relation between current strength and height of spike probably is due, at least in part, to variations in the extent of injury inflicted during the making and handling of the delicate preparations. As has been mentioned, this is indicated by the fact that statistically the ratios of the tangent of the

* Additional references relative to this subject are given by Huxley and Stämpfli.

angles formed with the base line of the anode and of the cathode lines are definitely smaller, on the average, with monophasic than with diphasic recording. Damage could have this effect if it diminished the rectifying action of the membrane. The much greater uniformity of tangent ratios derived from observations on the sturdier fresh sciatic nerve is in keeping with this view. It is a matter of some interest, however, that nerve fibers appear to be functioning quite normally through a range of rectification, such as is represented by tangent ratios of 1, or no rectification, and 3.6.

Stray demarcation currents, such as might result from local damage or from cut branches, cannot be a factor in this connection since any such would add themselves algebraically to the applied currents and the only result would be to shift the graphs laterally, so that the angle would no longer be at the zero of the applied current. Only one probable instance of this has been seen. That stray demarcation currents are not a main factor, if they are a factor at all, in the production of the angle is demonstrated by the presence of the angle in the experiments with fresh frog sciatic nerves. Indeed, it is worthy of note that in diphasic recordings the averages of the tangent ratios derived from multifibered (1.84) and single fiber (2.20) preparations are essentially alike. This is all the more remarkable in view of the fact that the multifibered spike is the average of the spikes from a wide range of fibers whereas the single fiber spike is probably that of a fiber larger in diameter than the average.

Tasaki *et al.* (18) have recently investigated the modification of the electric response of a single node of Ranvier by polarization. They, as we, used a resistance-capacity coupled amplifier. According to them, cathode polarization decreases spike height markedly, while anode polarization increases it, but only slightly. These results, they point out, and correctly, differ from those of all other investigators, and they ascribe the "discrepancies . . . to the complex character of the electrical network in the nerve trunk, especially to the highly polarizable character of the myelin sheath of the fiber in question or of the neighboring fibers". It seems more likely that their results differ from ours because of differences in networks and also because of differences in the time and the place of application of the currents employed. In their experiments the cathode of the stimulating circuit and the proximal recording and polarizing electrodes were one. The polarizing current was started only 11 msec. "before the onset of the long rectangular current pulse by which the spike to be recorded, was evoked." The duration of their stimulating current is not stated in this particular connection, but earlier in the paper they speak of "a rectangular current of 10 msec. duration." Under such conditions the stimulating current would add to the effect of the polarizing current when the latter was cathodal, and subtract from the effect of the polarizing current when it was anodal. It is just in these respects that the results pictured in their graph (their fig. 2 C) differ from ours: on the anode side of their graph the slope is slight (the increase in spike height is slight), whereas on the cathode side there is a steep decline. Their graph on the anode side seems to be concave upwards, but not clearly so because of the narrowness of range of amplitudes. On the cathode side their curve opens downwards, but the scattering of points is quite wide; consequently it is almost possible to regard their total graph as being composed of two straight lines meeting with an angle at zero, but with a *downward* bend there.

As to differences in the time relations of current application, their polarizing current, as stated above, starts only 11 msec. before the onset of the rectangular stimulating current through the common electrode. In our experiments the action potential is conducted to the polarized locus and arrives there several seconds (not msec.) after the start of polarization. But whether these temporal differences also are a factor in the production of the results they get, it is impossible to say.

The plotted data of our study yield curves of three types: *a*) There is the single-fiber type, linear on either side of zero up to polarizing current strengths that begin to deform the spike, or that actually block the impulse; this occurs at spike heights that range widely, between 118 and 182 per cent of the normal under anode polarization and between 19 and 60 per cent under cathode polarization. This type is not altered by storage of the preparation in Ringer's solution. Then there are multi-fibered types of two kinds. *b*) With fresh preparations the result again is linearity on either side of zero, but through a narrower range of change in spike height, the ultimate gradual departure from linearity occurring at heights of 113 to 133 per cent with anode polarization and 74 to 84 per cent with cathode polarization; i.e., at levels corresponding roughly with the lower levels of departure from linearity in the case of single fibers. *c*) With multifibered preparations stored 24 hours or longer the middle portions of the curves approach the shape of a reversed S, passing through zero almost linearly, i.e., without a sharp change in direction there, simulating in shape the curves of electrotonic potential published by Lorente.

Curves of type *a* could signify that the action of the current on a normal fiber is exerted primarily at a specific, the most accessible, locus until block supervenes there, that then the action of the current is displaced to the next, a more remote, most accessible locus, and so on. These loci might be either nodes of Ranvier or entire internodal segments. Curves of type *b* then would be the resultant of a multitude of curves of type *a*. And the disappearance of loci of higher susceptibility to current action, such that all parts of each fiber become equally susceptible, would result in curves of type *c*.

Possibly relevant to this view is the observation of Weddell and Glees (19) that in warm-blooded animals section of a nerve *in situ* results in structural changes in the myelin sheath that become obvious within 12 hours. Such changes might render the axon more accessible to applied currents and equally so everywhere. If this occurred within 24 hours in the case of the stored sciatic nerve of the bullfrog, curves of type *c* would be accounted for, since under these conditions spike height would change logarithmically with changes in the strength of the polarizing current. A much longer period of storage would be required to produce these changes in the case of the toe nerve because, due to its tenuousness, there would be less interference with its metabolism under these conditions.

There have been occasional variations from the rules as stated above. For these we have no explanation to offer. Our experience in this respect is not unique. Lorente, for example, notes that exposure to a given concentration of CO₂ may yield values in different nerves that differ by as much as 50 per cent under apparently like conditions (2).

SUMMARY

Determinations have been made of the effect of polarization on the height of the spike at the polarized locus, after amplification with a resistance-capacity coupled amplifier, of single medullated fibers of the phalangeal nerve and of preparations split from the sciatic; and, for purposes of comparison, similar observations have been made on multifibered responses from the unbranched part of the sciatic nerve of the frog, both freshly dissected and after storage in Ringer's solution at 5° C.

The height of the single fiber spike, with very few exceptions, increases linearly under anode, and decreases linearly under cathode polarization up to current strengths that produce signs of, or actual, block. The proportionality factor rather regularly is greater (but never smaller) for inwardly directed currents. The ratios of these factors range rather widely from preparation to preparation, due, possibly in part, to varying amounts of local damage done during preparation, though no sign of altered functioning is apparent even when this evidence of rectification is absent. The height of the spike in different preparations at the limits of linearity, in percentage of the normal, has ranged between 118 and 182 with anode, and between 19 and 60 with cathode, polarization. Storage of this preparation at 5° C. up to 43 hours does not alter the result appreciably. The evidence that we are dealing with the responses of single fibers is reviewed.

With the freshly prepared sciatic nerve the relation on either side of zero polarization likewise is linear in a majority of the cases. In such cases the range of rectification, as measured by the tangent ratios of the slopes to either side of zero, is relatively narrow, but the departures from linearity of the relation of spike height to polarization strength begin at relative spike heights that are roughly the same as the lowest of the values derived from single fibers.

Sciatic nerves stored 1 to 8 days regularly yield curves of the current strength vs. spike height which in their mid sections are similar in configuration to published curves of the relation of electrotonic potential to strength of polarizing current; they are reversed S in shape, pass through zero polarization without an angle, but with ordinates that are somewhat larger with ingoing than with outgoing currents.

These results are consistent with the view that in a normal, segmented, peripheral nerve fiber all of the susceptible processes that are concerned with the determination of the height of the spike are altered linearly by applied currents up to strengths that block, or begin to block, the nerve impulse, and that this relation breaks down in nerves stored under relatively anaerobic conditions, due possibly to a change in the dielectric properties of the myelin sheath.

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EFFECTS OF CHROMATOLYSIS ON INTERACTION OF SPINAL MOTONEURONS¹

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RENSHAW (1) has shown that antidromic volleys condition the synaptically excited discharges of other spinal motoneurons. The most striking effect is a pronounced inhibition when the synaptically excited cells and the motoneurons receiving the antidromic volley supply the same muscle or muscle group. Thus, conditioning effects are most apparent when the two groups of motoneurons occupy the same portion of the ventral horn, axially as well as cross-sectionally. The mechanisms involved in this conditioning have been reduced to the two most likely possibilities. These include the external action potentials of the antidromically fired cells and the impulses discharged by the recurrent collaterals transmitting the antidromic volley. Axonal chromatolysis of spinal motoneurons selectively reduces the external action potential without affecting conduction along the axons or, presumably, their collaterals (2). In this experiment, the chromatolysis of the conditioning motoneurons was used to differentiate between the two conditioning mechanisms that were postulated.

In Renshaw's experiment, a proprioceptive or monosynaptic reflex discharge was used as a test volley in conjunction with an antidromic conditioning volley. A significant feature of this conditioning was the early onset of inhibition of the test response. In fact, conditioning occurred when the testing impulses, which fired the tested motoneurons after a single synaptic delay, and the antidromic volley arrived in the ventral horn simultaneously. Therefore, indirect inhibitory mechanisms were eliminated as a source of conditioning because they require a longer conditioning interval than was present in that experiment. Renshaw (1) and Lloyd (3) described the experimental conditions that were most favorable for the production of maximum inhibition. These included the use of a maximal conditioning shock and a threshold proprioceptive test discharge. A fixed antidromic volley inhibited the greatest percentage of the total neurons fired when the test shock was near threshold. As the strength of the test discharge increased beyond this point, a fixed antidromic volley inhibited an increasingly smaller percentage of the total neurons fired. On the other hand, a constant threshold test response showed a progressive decrease in amplitude as the strength of the conditioning discharge was increased. But at the point of maximum conditioning, an increase of twenty-fold in the magnitude of the conditioning shock did not increase the response deficit.

The selective functional alterations produced by axonal chromatolysis were studied by two groups of investigators. Acheson, Lee and Morrison (4) found a progressive decrease in the amplitude of the spontaneous discharge recorded from the central end of a previously cut phrenic nerve, as compared to the uncut nerve on the opposite side. This effect became apparent 8 days after section, and increased in intensity up to 21 days. In critical animals, both phrenic nerves were excised, after definitive experimentation, and studied in a moist chamber. When correction was made for con-

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duction velocity, there was no significant difference in the amplitude of the maximal spike for A fibers in the two nerves. It was concluded that the deficiency in the spontaneous firing of the previously sectioned phrenic nerve was not due to failure of conduction in the phrenic axons.

Campbell (5, 6) correlated the physiological alterations of sciatic nerve and ventral root section with the resultant chromatolysis of the nerve cells in the lumbosacral cord and spinal ganglia of cats and monkeys. He found a loss of the proprioceptive component of the segmental spinal reflex during the period of chromatolysis. This did not stem from degeneration in the sensory system, for there was no alteration in the amplitude or rate of conduction from the peripheral to the central fibers of the dorsal root ganglion cells. Furthermore, the negative cellular response of the antidromic cord potential was decreased or absent, whereas the positive axonal potential of the approaching impulse was unchanged (2). Conduction along afferent pathways and axons of involved spinal motoneurons thus was not affected in chromatolysis. In further studies, Campbell, Gasteiger and Mark (7) quantitated the effect of chromatolysis in the multi-synaptic tibial-peroneal reflex. They found a decrease in the total electrical excitability of the reflex beginning 3 days after peroneal nerve section. The time course of this altered response in the early stages of chromatolysis was paralleled by histological studies of the involved spinal motoneurons. It was concluded that the chief functional alteration found at the spinal level was an increase in the threshold of excitability of the chromatolyzed motor nerve cells.

MATERIALS AND METHODS

Six cats had the nerves to their right semitendinosus and semimembranosus ligated and severed with sterile precautions and under light sodium pentobarbital anesthesia, 5 to 9 days before the experiment. The terminal procedure was carried out under very light dial anesthesia which was augmented by midthoracic spinal cord transection. Dorsal roots from L-5 to S-4 were severed intradurally and bilaterally. Reflex proprioceptive discharges were produced in the nerves to the biceps femoris by stimulation of appropriate dorsal roots. Antidromic discharge was initiated by stimulation of the nerves to the semimembranosus and semitendinosus. Recordings of the antidromic cord potential were made from the mid-dorsum of the cord with a monopolar ball electrode. The usual differential amplifier, sweep synchronized thyatron stimulator and cathode ray oscillograph were used. Histological sections were taken from the lumbosacral cord.

RESULTS

Both histological and physiological evidence of chromatolysis were present in the motoneurons of the nerves to the right semitendinosus and semimembranosus. Tigrololysis of motoneurons on the right was present at the S-1 level of a 9-day animal. The location of the chromatolyzed motoneurons, in cross-section, correspond to similar studies of the hamstring nuclei in dogs by Marinesco (8). The degree of chromatolysis resembled stage III of Campbell and Novick (9), with widespread dissolution of the Nissl substance and eccentricity of the nuclei. Reflex segmental discharges into the nerves of the right semitendinosus and semimembranosus were characterized by a delayed response without a discharge at the usual time of the proprioceptive deflection. In addition, the negative cellular component of the antidromic cord potential was deficient on the right. This is demonstrated in figure 1*a*, contrasting the right and left antidromic cord potentials in a 5-day animal with the same recording conditions.

The conditioning of a threshold proprioceptive reflex in the nerves to the biceps by a maximal antidromic volley initiated in the central end of the previously cut

nerve of the semitendinosus and semimembranosus was a pronounced inhibition in every case. In table 1 the maximum inhibitory effect is listed in 6 chromatolysis ex-

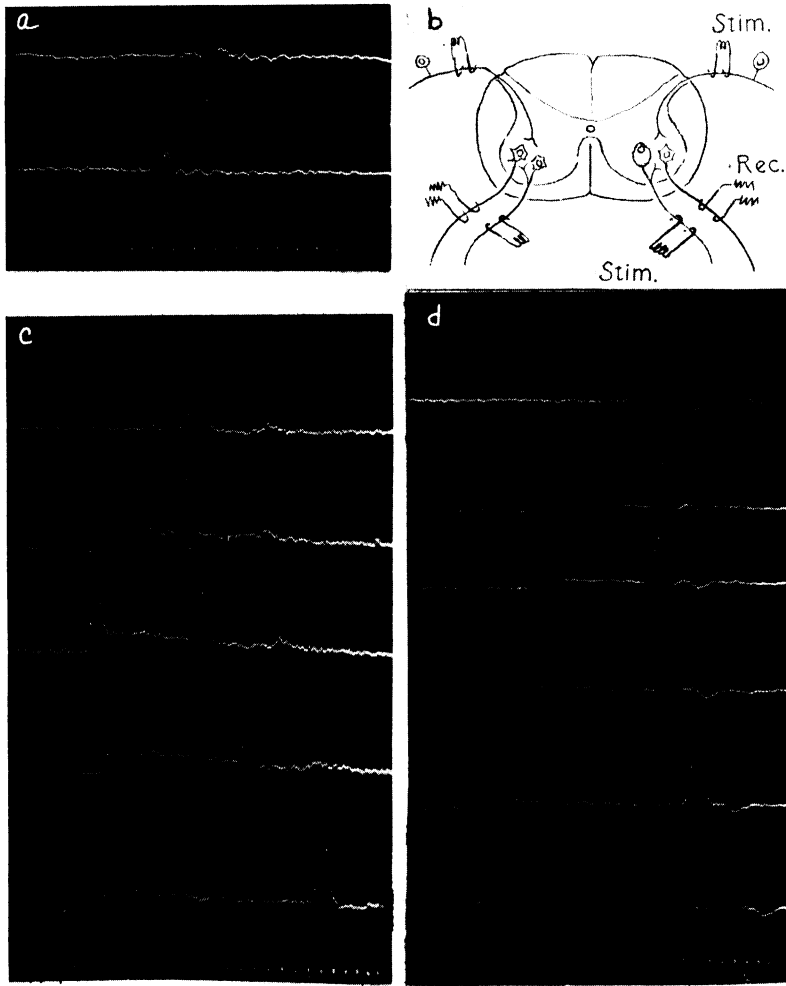


Fig. 1. *a.* COMPARISON OF ANTIDROMIC CORD POTENTIALS of chromatolysed (upper) and normal (lower) evoked by stimulation of the nerves to the semimembranosus and semitendinosus muscles. *b.* Schema of conditioning experiments. *c.* Conditioning effect of antidromic stimulation of normal neighboring neurons upon reflex discharge in the nerve to biceps femoris. *d.* As in *c* but with antidromic shock to chromatolyzed neurons. Time in milliseconds.

periments and compared to 5 normal experiments from this series. The maximum inhibition produced in the 6 experiments with chromatolyzed conditioning motoneurons varied from 25 to 52 per cent of the test response. The maximum inhibition in 5 control experiments varied from 25 to 56 per cent of the test response. The similarity in maximum conditioning effects is further illustrated by the oscillographic

records of the critical conditioning intervals on the control and chromatolyzed sides of a 5-day animal (fig. 1 *c, d*). These records also indicate a similar time course of inhibition on the two sides. This fact is borne out by the conditioning curves of 4 chromatolysis experiments contrasted with 2 normal conditioning curves in figure 2. Each significant point in each curve was the resultant average of between 10 and 24 alternate determinations of the conditioned and test response. The curves demonstrate the maximum biological variation obtained in the control and chromatolysis experiments. In both the chromatolyzed and the normal conditioning curves, a response deficit was present at an interval of 2 msec. These curves resemble the conditioning curves of similar experiments using the nerves to the 2 heads of the gastrocnemius by Renshaw (1) and Lloyd (3). In summary, the table of maximum inhibitions and the conditioning curves show a marked similarity in the chromatolysis and control experiments. This indicates that chromatolysis and the subsequent reduction of the external action potential of the conditioning motoneurons does not affect the magnitude or the time course of this conditioning.

TABLE 1. MAXIMUM INHIBITION LISTED AS PERCENTAGE OF TEST SHOCK

DAYS OF DEGENERATION	CHROMATOLYSIS EXPERIMENTS \bar{C}_0	CONTROL EX- PERIMENTS \bar{C}_0	DAYS OF DEGENERATION	CHROMATOLYSIS EXPERIMENTS \bar{C}_0	CONTROL EXPERIMENTS \bar{C}_0
5	38 ¹	52 ¹	9	25 ¹	
9	41	25	8	52	
5	52	54			58 ¹
8	45				30 ¹

¹ Maximum conditioning recorded only.

DISCUSSION

The recurrent collaterals of axons of mammalian spinal motoneurons were first mentioned by Golgi in 1883 (10). Their finding was substantiated by Ramon y Cajal (11), v. Koelliker (12) and others. According to v. Lenhossek (13), the majority of the collaterals originated from the axons as they passed from the gray to the white matter. The recurrent course of the collaterals was described by Ramon y Cajal (14). He followed these fibers to their apparent termination in the ventral horn in the vicinity of neighboring motoneurons. He was not able to give an exact description, however, of the termination of the collaterals on cell bodies or dendrites.

The functional significance of the recurrent collaterals of spinal motoneurons was discussed by v. Lenhossek in 1895 (13), who thought that they might influence the excitability of neighboring nervous elements. T. Graham Brown (15) tried to incorporate the collaterals into his theory of antagonistic half centers, as mediators of inhibitory impulses. A. Forbes (16) postulated a similar rôle for the recurrent collaterals in the reciprocal innervation of antagonistic muscles. Later, this theory was put to a careful experimental test (17). It was found that a crossed-extensor reflex, set up by contralateral sciatic nerve stimulation, was not modified by antidromic volleys arriving at the cord in motoraxons of the ipsilateral peroneal nerve. Renshaw (1) was the first to demonstrate the direct inhibitory effect of an antidromic volley on the reflex discharge of neighboring motoneurons, and he considered this effect a

mechanism for synchronization of the firing of motoneurons in a particular nuclear group.

Grundfest (18, 19) and Renshaw (1) have reviewed the biological effects of intrinsically generated action potentials on neighboring or contiguous nervous elements. The reduction of the external action potential in the present experiment, however, had no effect on the conditioning observed. Thus, in Renshaw's experiment, the possibility that conditioning effects were caused by the external action potential of the antidromically activated motoneurons is eliminated. The other inhibitory mechanism that was postulated, namely, the impulses discharged by the recurrent collaterals, could not be subjected to selective experimental evaluation in the spinal

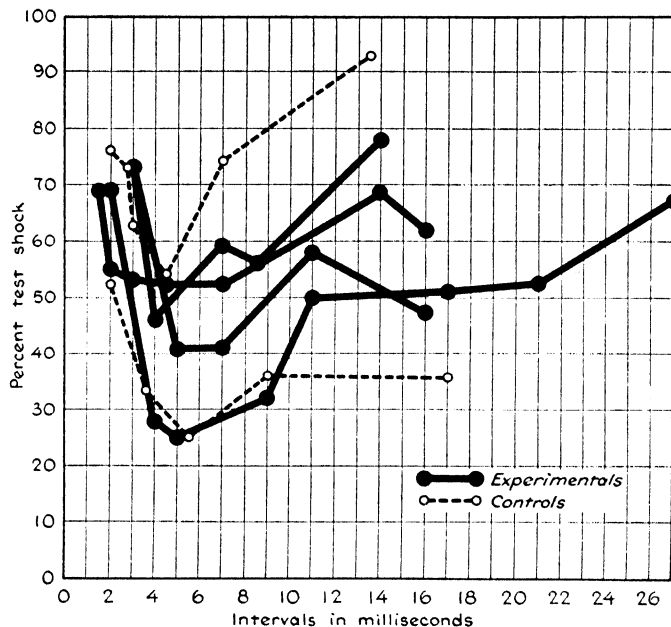


Fig. 2. Condition curves of 2 normal and 4 chromatolytic sides.

cord. Hence, only evidence of a suggestive nature is presented to implicate the recurrent collaterals as mediators of conditioning impulses.

SUMMARY

The conditioning of a proprioceptive discharge by an antidromic volley, in closely related groups of spinal motoneurons, was not altered by chromatolysis and the subsequent reduction of the external action potential of the conditioning motoneurons. This is direct evidence that the external action potential of the antidromically fired nerve cells does not exert a demonstrable conditioning effect on closely related groups of spinal motoneurons. This also suggests that the recurrent collaterals may be implicated as mediators of conditioning impulses.

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EFFECT OF CORTICAL STIMULATION ON RESPIRATORY RATE^{1,2}

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DURING the course of an investigation of cortical autonomic representation in dogs and cats (1), respiratory changes were observed almost invariably in association with autonomic responses. Because of the possible reflex effects of respiratory alterations on the autonomic functions under consideration (e.g. gastrointestinal motility) respiration was recorded in all experiments. With the appearance of the phenomena to be described, it became necessary to carry out a separate study designed to elucidate the effect of various anesthetic agents and stimulus characteristics on cortically induced respiratory changes, as well as to determine the cortical respiratory areas and the types of responses capable of being produced therefrom.

The literature on respiratory responses from the lateral and orbital surface has been reviewed by Delgado and Livingstone (2) recently and the reader is referred to their table 1 for a summary. There is general agreement among the recent workers that there is an area for acceleration of respiration in the sensory-motor cortex, and an area for inhibition and arrest on the orbital surface. Recently both acceleration and arrest of respiration have been reported from the cingulate gyrus (3).

METHODS

Experiments were performed on 10 dogs and 4 cats, all adults. Anesthesia was obtained as follows: *dogs*: 7-chloralose and urethane (1:10), 5-nembutal, 4-dial, 3-pentothal; *cats*: 3-dial, 1-2 per cent novocaine locally. Thoracic and abdominal excursions were recorded by a closed air system from a balloon girdle or rubber bellows apparatus attached outside the thorax and abdomen. Diaphragmatic movements were recorded from a balloon placed high up in the fundus of the stomach under the diaphragm. To eliminate abdominal wall artefact in the records of diaphragmatic respiration, the abdomen was incised widely transversely, the intestines retracted in a moist towel, and the temperature of the whole kept constant at 38-40 degrees. Blood pressure and pulse rate were also recorded, as well as many simultaneous records of gastrointestinal movements. All records were preserved on a continuous ink-writing kymograph.

The orbital surface was exposed by removal of the orbital contents. The cingulate gyrus was exposed by clipping or coagulation of the veins entering the longitudinal sinus, and retraction or removal of the hemisphere on the same side. This provided an adequate exposure of the opposite cingulate gyrus. The cortex was stimulated with bipolar silver wire electrodes of 2 to 3 mm. separation held in a ball and-socket clamp which permitted stability and easy mobility as desired. The points stimulated were recorded on a separate brain chart, or by photographing numbered tags placed directly on the cortex.

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³ N.R.C. (Can.) Fellow in Medicine 1947-48.

The stimulus was of the square wave type with independently variable frequency, intensity and wave duration to the extent that each wave could occupy 80 per cent of the interval between successive waves without distortion. An exploratory stimulus of 1 to 6 volts, 60 cycles and 6.4 milliseconds was used, since it was found to give most consistent results.

RESULTS

Chloralose and Urethane. The predominant response from stimulation of the cortex of dogs under chloralose and urethane was acceleration of respiratory rate. This effect was produced most readily from a point about 5 mm. anterior to the lateral end of the cruciate sulcus (fig. 1A). Less marked responses could, however, be

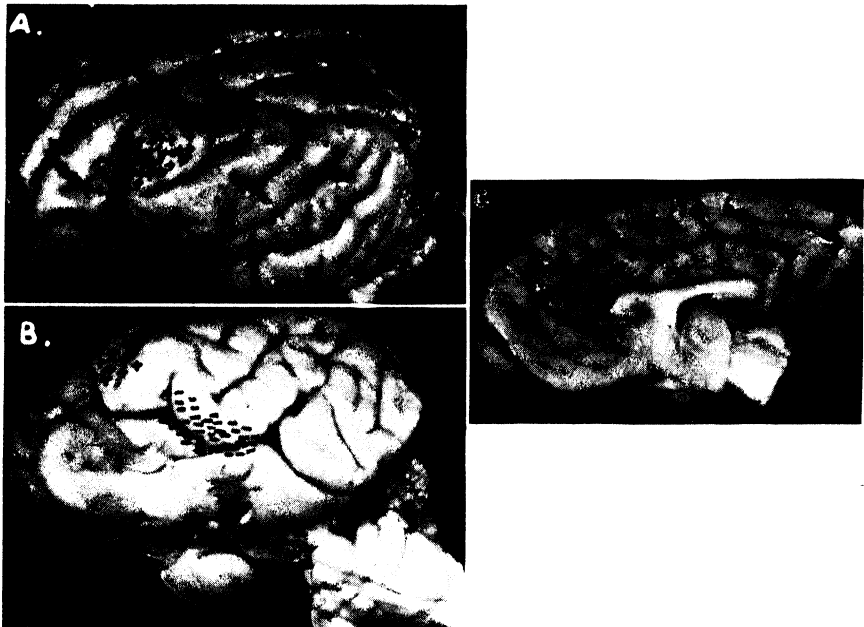


Fig. 1. DOG BRAIN. Alterations of respiratory rate from cortical stimulation under barbiturate narcosis or local anesthesia. A, lateral surface; B, orbital surface; C, medial surface. + = respiratory acceleration; - = respiratory slowing or arrest.

produced from the anterior and posterior sigmoid gyri, the anterior end of the coronal, ectosylvian and sylvian gyri and an irregular strip of cortex anterior to the presylvian sulcus and lateral to the olfactory tract. Medially, acceleration occurred from stimulation of the entire middle and anterior portions of the cingulate gyrus.

Slowing of respiration using chloralose and urethane was seen in only two experiments, the area for the response being the anterior end of the ectosylvian gyrus (fig. 1B). Arrest of respiration was never seen in the dog under this type of anesthesia. No inhibitory effect on respiration could be obtained from the sub-genual portion of the anterior cingulate gyrus with this anesthetic.

A heretofore unreported phenomenon was produced at a very light level of chloralose and urethane anesthesia (fig. 2). This consisted of an increase of rate, a

diminution of the thoracic excursion and an increase in the diaphragmatic excursion during stimulus. Although the stimulation lasted only 10 to 20 seconds, there was a gradual decrease of the thoracic excursion until in 10 to 100 seconds the thorax came to rest in the expiratory position, and the diaphragm alone continued to perform the respiratory act. Observation of the animal at this time showed completely flaccid thoracic and abdominal walls, the abdomen moving only passively due to the diaphragmatic contractions.

The full 'shift' persisted for 30 to 60 seconds when, by the same gradual process, thoracic respirations gradually increased in amplitude and the diaphragmatic ex-

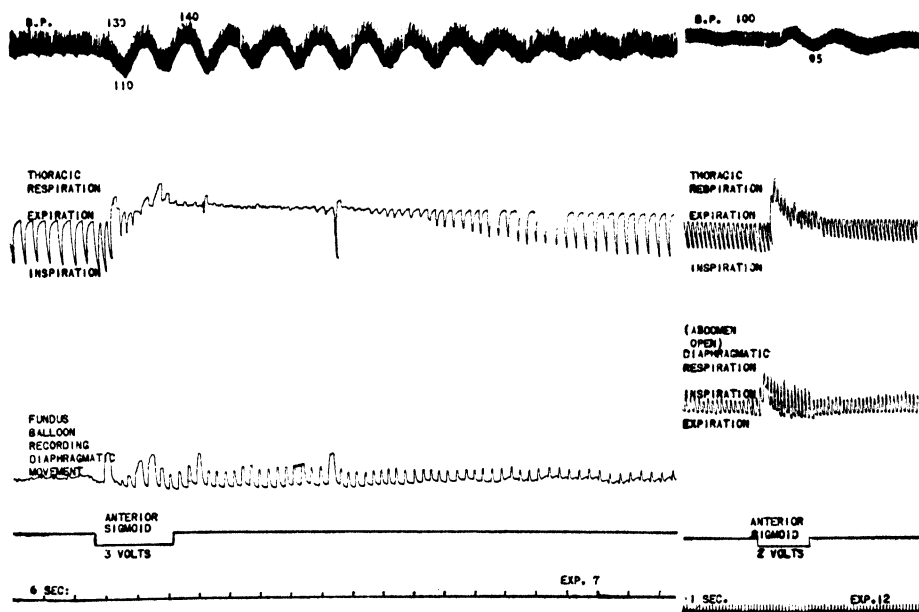


Fig. 2 (left). 'SHIFT' OF RESPIRATION from predominantly thoracic to predominantly diaphragmatic, from stimulation of the lateral end of the anterior sigmoid gyrus.

Fig. 3 (right). SLIGHT ACCELERATION OF RESPIRATION with decrease of thoracic excursion and increase of diaphragmatic excursion, but without complete 'shift' from same point as figure 2 with weaker stimulus.

cursions diminished until the original conditions were reestablished 2 to 3 minutes after stimulation. This response could be reproduced at will from those areas of the cortex which gave an augmentation of respiration in any given experiment. It was also elicited (as were the other changes herein described) as a reflex response to certain types of peripheral stimulation.

The chief factor in the production of this phenomenon was a light level of chloralose and urethane anesthesia. Deepening the anesthesia or reducing the strength of the stimulus eliminated the second part of the response, so that slight acceleration with diminution of thoracic excursion and increase of diaphragmatic excursion occurred, and was confined to the period of actual stimulation; the 'after effect' did not appear (fig. 3).

Barbiturates. The depression of autonomic reflexes produced by the dosage of barbiturates sufficient to inhibit somatic reflexes seemed to be much greater than that resulting from similar dosage of chloralose and urethane. Nevertheless, the following responses were not specifically due to effects of anesthesia since they could be produced from the same areas in unanesthetized preparations.

The changes in respiratory rate from cortical stimulation were qualitatively similar for all the barbiturates and are summarized in figure 1. With this group of anesthetic agents there are four well defined cortical areas from which changes in respiratory rate may be produced:

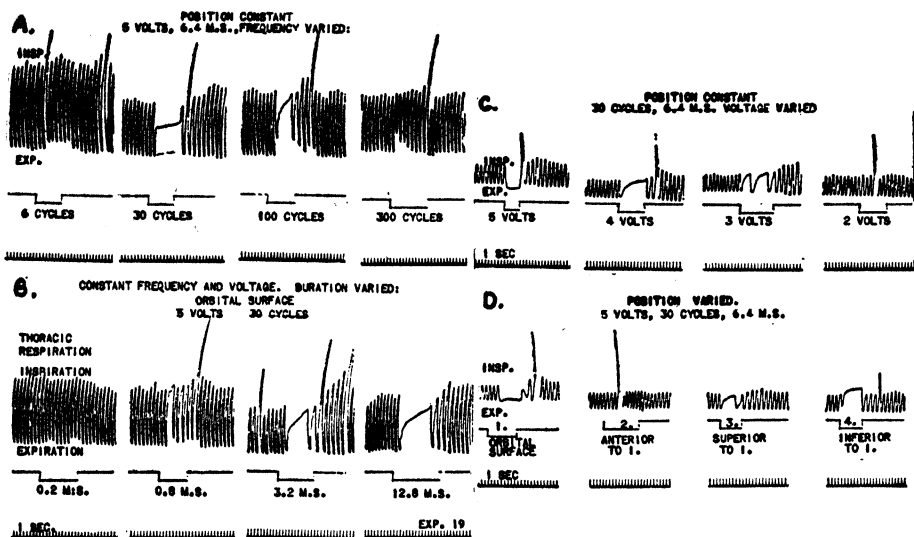


Fig. 4. ANALYSIS OF STIMULUS CHARACTERISTICS for respiratory inhibition from the insular-orbital region. A, frequency varied; B, wave duration varied; C, intensity varied; D, position altered. Note apparent acceleration following gasp in D-2.

Acceleration: a) centering around the lateral end of the anterior sigmoid gyrus and extending anteriorly to and across the presylvian sulcus (fig. 1A); b) the middle and most of the anterior portions of the cingulate gyrus, with the exception of b below (fig. 1C).

Inhibition: a) roughly defined as the 'insular-orbital region'⁴ which includes the anterior ends of the sylvian and ectosylvian gyri (anterior composite gyrus) and the adjacent parts of the frontal lobe and olfactory tract (fig. 1B); b) a small area on the anterior cingulate gyrus anterior and inferior to the genu of the corpus callosum (fig. 1C).

Using a higher speed kymograph it could be shown that the latent period for

⁴ This term is used to emphasize the homology between the sylvian and anterior sylvian gyrⁱ of animals and the anterior part of the Island of Reil in man (4). While the entire area is in direct relationship to the roof of the orbit, the term 'orbital surface' should be reserved for the orbital gyri and other portions of the inferior surface of the frontal lobe.

these responses was dependent upon the phase of the respiratory excursion during which stimulation began. For example, inhibition in the cat, occurring maximally in expiratory arrest (fig. 4C), appeared only at the conclusion of the respiratory cycle during which the stimulus was applied. The alteration of rate could be maintained for a variable period, depending on its intensity. Complete arrest was seldom prolonged beyond 10 seconds, when escape occurred at a slower rate and diminished amplitude. With cessation of stimulation, the original rate was usually resumed immediately, although there was occasionally a short after effect.

Stimulus Characteristics. Acceleration responses were more readily produced in the dog, while inhibition was most consistently apparent in the cat. For this reason the following analysis of the quantitative and qualitative effect of stimulus characteristics on the cortically induced respiratory inhibition was carried out on the cat. Optimal frequencies lay between 30 to 60 cycles per second (fig. 4A), while above 200 cycles per second and below 10 cycles per second there was either no change or, occasionally, slight acceleration. Optimal wave duration with this type of stimulator was 6.4–12.8 m.s. with an effective range of 3.2–25.4 m.s. (fig. 4B). Beyond that range the inhibition was submaximal, but acceleration never appeared as long as the intensity and frequency were not altered. The phase during which stimulation was applied bore no relation to the phase of arrest. By altering the intensity it was possible to show that the maximal response consisted, in the cat, of arrest in expiration (fig. 4C) due to inhibition of the inspiratory cycle. The same method could be used to determine the 'center' of the responsive area (fig. 4D).

A similar analysis of the stimulus characteristics for acceleration revealed wider variations of the same order as for inhibition. However, while it was occasionally possible to produce acceleration from the so-called inhibitory areas using stimuli outside the mentioned ranges or very light anesthesia, it was not possible to produce respiratory arrest from the so-called accelerator areas.

Blood Pressure. Blood pressure responses were not studied independently of respiration but an analysis of all the changes in this series with chloralose and urethane anesthesia showed that the usual response was depressor from all the respiratory areas. This was usually more marked when there was an alteration of respiration and seemed to be greatest from the sub-genua portion of the anterior cingulate gyrus, where the fall sometimes amounted to 60 mm. On the other hand, with the barbiturates there was frequently a slight rise of the blood pressure at the beginning of stimulation (particularly from the orbital surface), followed by a somewhat greater fall if respiratory inhibition occurred, and finally a slight rise following the end of the stimulus, before the original conditions were reestablished. Since these alterations were more marked when there were also respiratory changes, it was felt that they were at least partly cardio-respiratory reflexes, rather than indicative of specific cardio-vascular centers.

It is interesting in this regard that the blood pressure response from the anterior cingulate gyrus could be reversed by cutting the vagi. Prior to bilateral vagotomy a fall of 30 mm. occurred from stimulation of this area, whereas after vagotomy and the introduction of artificial respiration a rise of 30 mm. was produced by stimulation of the same point.

DISCUSSION

The influence of different anesthetic agents on respiratory responses from the cortex was first discussed by Spencer (5) who felt that the level of anesthesia, as well as the type of anesthetic used, determined the quantitative and, to some extent, the qualitative variations in results. The results presented here bear out this criticism of much of the past and current work in the field of autonomic responses from the cortex. It must be concluded that no blanket statement can be made regarding the type of response obtainable from any given cortical area, unless and until the effects of the anesthetic agents on the cortical and subcortical neuronal mechanisms, and the effector organs, are known. Similarly, full account must be taken of the interactions of the various changes in different systems (e.g. B.P. and respiration) which are altered by stimulation of the same area. To a lesser extent we must also begin to appreciate the influence of variations in stimulus characteristics.

We have confirmed previous workers regarding the presence of respiratory acceleration area about the lateral end of the anterior sigmoid gyrus and presylvian sulcus, and an inhibitory area in the 'insular-orbital' region (not from the coronal gyrus, 6). The respiratory responses from the cingulate gyrus have been clarified. The latter has been divided into two functionally distinct portions, a large area for acceleration and a small, well localized area anterior and inferior to the genu of the corpus callosum for inhibition.

It has been shown that inhibition of respiratory rate may be produced by stimulation of the 'insular-orbital' surface in unanesthetized animals, similar in every respect to that occurring in animals under barbiturate narcosis. Therefore, the absence of 'inhibitor' area in dogs under chloralose and urethane is due to the level of anesthesia or some other action of these agents on the neuronal mechanisms involved.

The previously unreported phenomenon consisting of a shift from thoracic to diaphragmatic respiration similarly must be considered to be a non-specific response, since it was obtainable from any cortical respiratory accelerator area under the required conditions of anesthesia. It is not unlike the normal respiration in the relaxed and weary state, the respiration of deep sleep, or that of third stage anesthesia. It differed from the usual cortical motor responses in that it continued despite cessation of the stimulus. It was occasionally preceded by apparent hyperventilation, produced either by increased rate or depth of respiration, but could not be produced by hyperventilation alone. The importance of this phenomenon, then, lies not in the cortical area from which it can be induced (since this is diffuse) but in the fact that a disturbance of the balance between the two major effector muscle groups for the respiratory act can be produced by cortical stimulation.

It was not within the scope of this work to analyze the other properties of the respiratory act, such as inspiratory and expiratory 'tonus' (5) nor to make a detailed study of the variations of amplitude of the abdominal and thoracic components. Examples of these may be seen in figures 3 and 4. It is obvious, however, that these factors play a rôle equal to, if not greater than, the rate in the volume of respiratory exchange and will require eventual elucidation.

The effect of different frequencies of stimulation on respiratory responses induced by direct vagal stimulation has been reported (7, 8).

The analysis of the stimulus characteristics is not yet complete. However, the results presented indicate that there are limits to the electrical characteristics of the stimulus beyond which the cortical mechanisms will not respond. This will find explanation eventually in the electrical properties of the individual neuronal pathways. It may be, and we have suggestive evidence, that there are different parameters of electrical stimuli for different systems represented in the same cortical area.

Recently it has been suggested that an opposite effect on the same function may be produced by different frequencies of stimulation on the same cortical area (2). Although we have also seen occasional acceleration from the inhibitory areas of the orbital surface with barbiturates, this has not been consistently true. It seems more likely that this is what Wyss has called a non-specific response (8). However, as indicated by the reversal of blood pressure response to stimulation of the sub-genual portion of the anterior cingulate gyrus after bilateral vagotomy, it may be that there is a double representation of both sympathetic and parasympathetic systems in the one region (9). When the dominant system is abolished the opposite effect appears.

All the respiratory responses obtained by cortical stimulation can be obtained through peripheral reflex mechanisms (10). Arrest of respiration from the insular orbital region and from the sub-genual portion of the anterior cingulate gyrus is analogous to that obtained by vagal stimulation, through medullary centers. Acceleration from the anterior sigmoid and anterior and middle cingulate gyri is similar to that produced by stimulation of peripheral noci- and chemoceptors. Thus it is very probable that the cortical representation of respiration is simply a more specialized reduplication of lower level mechanisms, presumably allowing of more elaborate and integrated control.

The similarity of the 'vagal' motor effects on gastric motility and blood pressure from stimulation of the insular-orbital surface and the sub-genual portion of the anterior cingulate gyrus has been discussed in another paper (1). The addition of similar respiratory effects from the two areas is further evidence of a functional relationship between them.

SUMMARY

The effect of different anesthetic agents and stimulus characteristics on changes of respiratory rate by electrical stimulation of the cortex has been considered.

There are two areas for inhibition of respiratory rate in the dog and cat: *a*) a small area on the anterior part of the cingulate gyrus just anterior and inferior to the genu of the corpus callosum; *b*) a larger area designated the 'insular-orbital' region which includes the anterior part of the anterior sylvian and ectosylvian gyri, the posterior portion of the frontal lobe between the presylvian and anterior rhinal fissures, and the adjacent portion of the olfactory tract.

Acceleration of respiration in the dog and cat may also be produced from two separate areas: *a*) the anterior sigmoid and presylvian region; *b*) the anterior and middle portions of the cingulate gyrus, exclusive of the sub-genual area mentioned above.

The maximal stimulus characteristics for inhibition of respiratory rate from the

insular-orbital surface using a square wave stimulator have been outlined. A previously unreported phenomenon consisting of shift from predominantly thoracic to predominantly abdominal respiration is discussed, and a functional relationship between the insular-orbital surface and the sub-genual portion of the anterior cingulate gyrus is suggested.

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VARIATION IN ACETYLCHOLINE CONTENT OF THE BRAIN WITH PHYSIOLOGICAL STATE

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ACETYLCHOLINE is present in significant quantity in the mammalian brain. It appears to occur mainly in the form of a physiologically inactive complex which liberates free acetylcholine when the tissue is treated with extractants. The level in the brain *in vivo* must depend on *a*) the rate of synthesis, *b*) the rate of liberation from the complex and *c*) the rate of loss by enzymic breakdown or by diffusion from the tissues.

It has been suggested that acetylcholine may play a part in nervous transmission in the central nervous system. If nervous activity is associated with an increased liberation and breakdown of acetylcholine, the level in the brain *in vivo* might be expected to vary to some extent with the state of functional activity of the brain. The level should be maximal in conditions of reduced activity, as in anesthesia and sleep, while it might show a temporary fall in states of increased neuronal activity, as during convulsions. The present investigation was carried out to test if evidence could be found of any variation of the acetylcholine content with the degree of functional activity of the brain.

METHODS

Acetylcholine determinations were carried out on the whole brains of young Wistar albino rats of 20 to 30 gm. The animals were killed by immersion in liquid air, which produced a rapid fixation of any biochemical changes in the tissues and minimized post-mortem changes due to the breakdown or resynthesis of acetylcholine in the brain. This method of killing also appeared to cause less stimulation of the brain than other methods such as decapitation (1). The liquid air was put in a large wide-mouthed beaker so that the animals could be dropped straight in without delay.

Series of animals were taken for acetylcholine estimation: *a*) during anesthesia; *b*) while sleeping; *c*) in the normal waking state; *d*) during emotional excitement *e*) after electrical stimulation of the brain; and *f*) during convulsions.

Anesthesia was obtained by intraperitoneal injection of sodium pentobarbital (5 mg/kg.); the temperature of the animals was maintained by placing them on a warm metal plate. Animals were made to sleep by the warmth and light of an operating table lamp placed over the cage. Emotional excitement was produced by tipping from side to side in a large beaker for 4 minutes. Electrical stimulation of the brain was effected with 35 to 50v. A.C. at 50 cycles/second by platinum electrodes of 0.25 sq. cm. area applied to the shaved scalp 0.5 cm. posterior to the eyes. Electrical stimu-

lation for 1 to 3 seconds produced satisfactory convulsions after a usual latent period of about 10 seconds, during which the animals were in coma. The procedure was similar to that used in the electroshock treatment of psychiatric patients. Further details of these methods are given by Richter and Dawson (2, 3). A normal control was included with one from each experimental group in any particular series of determinations and littermates were used as far as possible.

Extraction of Acetylcholine. The validity of the results depended on obtaining a consistently high extraction of the total brain acetylcholine. The reliability of the usual methods has sometimes been questioned and special attention was therefore given to the method of extraction. The brain was dissected from the skull using chilled instruments and keeping the brain frozen by the use of further quantities of liquid air. It was then finely powdered in a cooled steel crusher. In order to minimize any errors due to incomplete extraction two different methods were used.

Method 1. Extraction with Buffered Saline. In this method the powdered brain was stirred at 0° C. into a mixture of 1 ml. acetate buffer pH 4.0 (made by diluting 100 ml. N acetic acid and 31 ml. N sodium hydroxide solution to 700 ml.) and 3 ml. eserized acidified amphibian Ringer-Locke solution. The Ringer-Locke solution before acidification contained 6.5 gm. NaCl, 0.14 gm. KCl, 0.2364 gm. $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.2 gm. NaHCO_3 per liter; it was brought to pH 4.0 with N/4.5 hydrochloric acid, using bromophenol blue, and 1 part in 4000 of eserine sulfate was added. The solution in a centrifuge tube was weighed before and after addition of the powdered brain. The mixture was rapidly brought to the boil on a Bunsen flame, allowed to cool, stirred with a glass rod and centrifuged for 5 minutes. The supernatant solution was decanted, the residue washed with 1 ml. eserized amphibian Ringer-Locke solution at pH 4 and the combined supernatant solutions were kept in stoppered graduated tubes in the refrigerator until required for assay.

Method 2. Extraction With Trichloroacetic Acid. In this method the powdered brain was stirred with 4 ml. 10 per cent trichloroacetic acid at 0° C. in a previously weighed centrifuge tube and the mixture was shaken thoroughly. The tubes were kept on ice for 30 minutes, the mixture was then centrifuged 4 minutes, the residue washed with 1 ml. 10 per cent trichloroacetic acid and the combined supernatant solutions brought to pH 4 with N sodium hydroxide solution. The solution was kept in the refrigerator until assayed. The presence of sodium trichloroacetate at the dilution used did not interfere with the estimation of acetylcholine by the frog rectus preparation.

In 6 experiments the powdered brain was ground with sand in a cooled mortar containing the extractant at 0° C.; this modification of the procedure produced no significant difference. In a further series of 9 animals the completeness of the extraction was tested by re-extracting the residue from the first extraction. Assays carried out on the second extract, of which 5 were made with buffered saline and 4 with trichloroacetic acid, gave a further small amount of acetylcholine; the figures were 0.04, 0.02, 0.01, 0.0, 0.08, 0.0, 0.04, 0.02 and 0.0 $\mu\text{g/gm.}$ acetylcholine. These figures indicated that the first extract, made by either *method 1* or *method 2* contained 95 to 100 per cent with a mean of 98 per cent of the total acetylcholine obtained in the two extractions. Further tests showed a high recovery of known amounts of acetylcholine added to the residue and then extracted by the normal procedures. In 6

experiments the amounts added were 2.0, 2.0, 1.0, 2.0, 2.0 and 1.0 $\mu\text{g.}$ acetylcholine; the amounts found by assay on extraction with buffered saline were 1.85, 1.9 and 0.95 $\mu\text{g.}$ in the first 3 experiments and 2.0, 1.9 and 0.97 $\mu\text{g.}$ on extraction with trichloracetic acid in the last 3 experiments, respectively. The close agreement in the figures obtained for the brain acetylcholine by the two different methods of extraction also gave evidence that the methods of extraction used were satisfactory (table 2).

Acetylcholine Assay. In a few preliminary experiments the leech muscle and cat blood pressure preparations were used, but in all the subsequent work the frog rectus method was preferred as it was apparently more satisfactory for the brain extracts which had to be tested. The frog rectus muscle was set up in a 4 ml. bath of amphibian Ringer-Locke solution. It was allowed to stand 2 hours in the oxygenated solution under a small tension of 0.75 gm. The solution was frequently changed during the first hour and for the second hour it was replaced by Ringer-Locke solution containing 1 in 100,000 eserine sulfate. Before starting the determinations the muscle was tested several times with a small amount of acetylcholine to make sure that its sensitivity was constant and that it always relaxed to the same length. If these conditions were not fulfilled the muscle was left for a further 30 minutes in eserinated Ringer-Locke solution and then re-tested. Most of the assays were carried out in an unheated laboratory during the winter months, but if the room temperature rose above 10°C. the bath temperature was reduced to 10° before use.

Immediately before assay, each brain extract was divided into two equal parts, A and B. Solution A was brought to $\text{pH } 7$ with sodium hydroxide solution and then diluted with amphibian Ringer-Locke solution so that each ml. contained the extract from 100 mg. of brain. Solution B was made alkaline ($\text{pH } 11$) with sodium hydroxide solution, boiled to destroy the acetylcholine, brought to $\text{pH } 7$ and diluted with Ringer-Locke solution to the same volume as solution A. Portions containing 1 ml. of solution A made up to 4 ml. with Ringer-Locke solution were then assayed against known amounts of acetylcholine chloride made up in 1 ml. of solution B and similarly brought to 4 ml. with Ringer-Locke solution. This method, due to Feldberg (4), avoided errors due to sensitizing substances present in the brain extracts. Acetylcholine solutions were made up daily from sealed phials containing 1 mg. acetylcholine in 1 ml. 10 per cent dihydrogen sodium phosphate solution. The eserinated Ringer-Locke solution was made daily by adding the calculated amount of solid eserine sulfate to a diluted stock solution.

By the described procedure the brain from a 30-gm. rat provided sufficient extract for 5 contractions of the rectus muscle and it was possible to bracket one or more of these between two closely approximating amounts of acetylcholine. The sensitivity of the rectus preparation under the conditions used was such that it would regularly detect as little as 0.02 $\mu\text{g.}$ of acetylcholine in the 4 ml. bath; it would detect differences of the same order, so that estimations of acetylcholine content could be made with an error of not more than 10 per cent. Results are expressed as $\mu\text{g.}$ acetylcholine chloride per gm. fresh brain tissue.

RESULTS

Preliminary Experiments. The substance present in brain extracts which causes contraction of the frog rectus muscle was shown to have the properties of acetyl-

choline in that: *a*) it was destroyed by heating with alkali at pH 11.0 for 1 minute; *b*) it was destroyed by incubating with cholinesterase prepared from red blood cells; *c*) it caused contraction of the leech muscle; and *d*) it produced a fall in the blood pressure, abolished by atropine, in the cat.

In a series of preliminary experiments, which are described in brief, the brain acetylcholine content of rats of 40 to 50 gm. was estimated with the leech muscle preparation. The acetylcholine was extracted with buffered saline (*method 1*) and assayed in the usual manner against a standard solution of acetylcholine in saline. In a further series of experiments carried out in collaboration with Dr. F. C. MacIntosh and Dr. R. M. C. Dawson the acetylcholine in extracts prepared with trichloroacetic acid (*method 2*) was estimated with the cat blood pressure preparation. Substances other than acetylcholine in the brain extracts tended to interfere with the acetylcholine response and, as in these preliminary experiments the animals taken during

TABLE 1. VARIATION IN ACETYLCHOLINE CONTENT OF RAT BRAIN

METHOD OF ESTIMATION	ANESTHESIA	SLEEP	NORMAL	EXCITED	CONVULSIONS
	$\mu\text{g./mg.}$	$\mu\text{g./mg.}$	$\mu\text{g./mg.}$	$\mu\text{g./mg.}$	$\mu\text{g./mg.}$
Leech muscle	2.9	2.22	1.9	1.4	1.7
	2.4	2.6	1.6	1.5	1.1
	2.8	2.3	1.3	1.6	1.1
			1.4		1.8
Mean	2.7	2.4	1.55	1.5	1.4
Cat blood pressure	2.8		1.7	2.0	1.6
	2.5		2.5	2.1	1.4
	2.2		1.8	1.9	1.5
			2.4	1.5	
			1.5		
Mean	2.5		2.0	1.9	1.5

convulsions were transferred to liquid air during the first 5 to 10 seconds of the convulsions, the maximum fall was missed; but by both methods of assay the relative acetylcholine values showed a general trend towards lower acetylcholine levels in states of greater activity (table 1). The acetylcholine content for anesthetized animals was 70 to 100 per cent higher than for animals taken during convulsions.

Normal Series. The acetylcholine content of the brains of 15 young rats of 20 to 30 gm., determined by the frog rectus method, gave a mean value of 1.25 $\mu\text{g./gm.}$ fresh brain tissue, with a range of 0.9 to 2.0 $\mu\text{g./gm.}$ and S.D. of ± 0.29 . This mean value was lower than some other figures given in the literature and lower than the mean for the preliminary experiments. This may be due in part to the special precautions taken to prevent the resynthesis of acetylcholine, which is rapid in nervous tissue (5), and in part to the use in these experiments of younger animals: it was shown by Welsh and Hyde (6) that the acetylcholine content of the rat brain increases with age. The method of assay which was used corrected for the presence of substances in the brain extracts which sensitize the rectus muscle to acetylcholine and

which tend to give unduly high figures for the acetylcholine content if they are not taken into account. The effect of such sensitizing substances varies with the particular brain extract and with the assay preparation, but it was found that under the conditions of these experiments the figure obtained for the acetylcholine content could be as much as 50 per cent above the true value if this source of error was ignored. Good agreement was obtained between the groups in animals in which the acetylcholine extraction was carried out by different methods (table 2).

Animals in the normal series were brought into the laboratory some time before killing and kept in a warm place. After a few hours under these conditions they be-

TABLE 2. ACETYLCHOLINE CONTENT OF RAT BRAIN IN DIFFERENT STATES OF ACTIVITY. FROG RECTUS METHOD

EXTRACTANT	ANESTHESIA	SLEEP	NORMAL	EXCITED	ELECT. STIMU.	CONVULSIONS
	$\mu\text{g/gm.}$	$\mu\text{g/gm.}$	$\mu\text{g/gm.}$	$\mu\text{g/gm.}$	$\mu\text{g/gm.}$	$\mu\text{g/gm.}$
Buffered saline	2.1	1.5	1.3	0.95		0.55
	1.9	1.3	1.3	0.75		0.40
	1.6	1.6	1.3	0.85		
			1.0	1.3		
			1.2	1.0		
				1.2		
				1.1		
Mean	1.87	1.47	1.22	0.99		0.48
Trichloroacetic acid	1.5	1.4	1.0	0.90	0.45	0.55
	1.9	1.8	2.0	0.85	0.50	0.50
	1.8	1.5	1.5	0.55	0.80	0.60
	1.9	1.0	1.2	0.53	0.55	0.68
	1.4		1.4	0.80	0.65	0.50
			0.95	1.3	0.30	
			1.5	0.95	0.65	
			1.0	0.40	0.65	
			0.9	0.60	0.42	
			1.2	0.84		
Mean	1.7	1.43	1.27	0.82	0.55	0.57
Overall mean	1.76	1.44	1.25	0.87	0.55	0.56

came quiet and they were transferred to liquid air while feeding or while moving quietly in their cage. The liquid air was contained in a wide-mouthed beaker and usually they entered it without any sign of being frightened, but any animal which was felt to struggle before entering the vessel was discarded. These precautions may account for the small standard deviation found in these experiments.

Effects of Anesthesia and Sleep. Eight animals lightly anesthetized with sodium pentobarbital gave a mean acetylcholine content of 1.76 $\mu\text{g/gm.}$ (range 1.4–2.1; S.D. 0.24) and 7 animals taken in the sleeping state gave a mean of 1.44 $\mu\text{g/gm.}$ (range 1.0–1.8; S.D. 0.25). Both of these values were higher than for normal animals and the differences were statistically significant ($P < 0.01$); they were also significantly differ-

ent, at the 0.05 level of probability, from one another. The result for anesthetized animals agrees with a previous report of Tobias, Lipton and Lepinat (7), who also found a higher brain acetylcholine content in anesthetized rats. The individual values obtained by them in both groups were somewhat higher than those obtained in the present series, but this may be due to differences in the experimental conditions and technique.

Emotional Excitement, Electrical Stimulation and Convulsions. A series of 17 rats, which had been excited for 4 minutes by the method of repeatedly removing their support, gave a mean brain acetylcholine content of $0.87 \mu\text{g}/\text{gm}$. (range $0.4-1.3$: S.D. 0.26). This was significantly lower than the normal mean. Lower values were also obtained for further series of animals taken during electrical stimulation of the brain

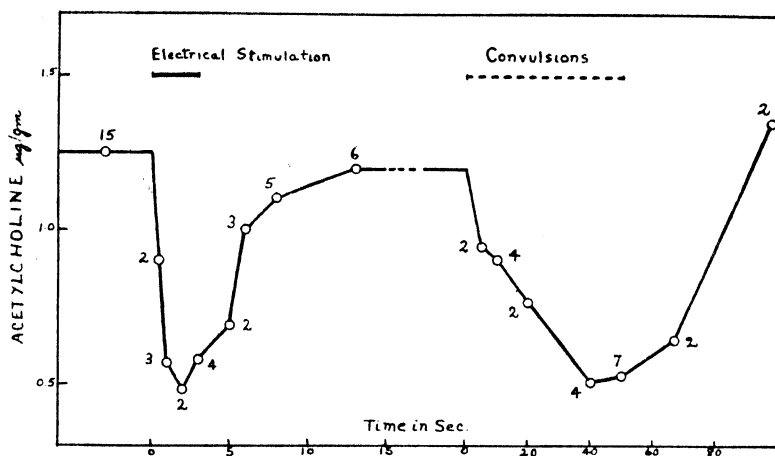


Fig. 1. SHOWING EFFECT OF ELECTRICAL STIMULATION and subsequent convulsions on the acetylcholine content of the rat brain. Each point gives the mean value for 2 or more animals, the number of which is given alongside. Electrical stimulation was given for 3 sec. except for animals killed after a shorter period of stimulation. The latent period before the commencement of convulsions varied from 8 to 15 sec. for different animals. The values during and after convulsions are therefore given on a separate time scale starting at the time when convulsions commenced.

and during convulsions. The differences from the normal mean were significant in each group at the 0.01 level of probability. The electrically stimulated animals, which were transferred to liquid air after passage of the electric current through scalp electrodes for 1 to 3 seconds, gave a mean acetylcholine content of $0.55 \mu\text{g}/\text{gm}$. with a range of 0.30 to 0.80 and S.D. 0.16 . The electrical stimulation under these conditions caused an immediate generalized muscular spasm, followed by a period of coma lasting for about 10 seconds. Convulsions usually started then and continued for about 40 to 50 seconds. The convulsions gradually became intermittent and then, often after a period of hyperactivity, the animals became relatively quiet and inactive. Animals taken after the convulsions had continued for 40 to 50 seconds gave a mean brain acetylcholine content of $0.56 \mu\text{g}/\text{gm}$. with a range of 0.4 to 0.68 and S.D. 0.09 .

Rate of Loss and Resynthesis of Acetylcholine in the Brain In Vivo. In order to study the rate of the change in the brain acetylcholine level, groups of animals were

taken for examination at various times during and after electrical stimulation and during the convulsions which ensued. The results for 65 animals, which are given diagrammatically in figure 1, showed that the sharp fall in the brain acetylcholine level during electrical stimulation was followed by a rapid rise. In animals taken after 1 to 3 seconds of electrical stimulation there was a loss of over 50 per cent of the total brain acetylcholine content. The times given in figure 1 refer to the time when the animal was transferred to liquid air. The transference, timed with a stop-watch, took 0.4 to 0.6 seconds. The time taken for the cortex of the brain to cool to 0° C., shown by means of a thermocouple, was approximately 4 seconds. A part of the acetylcholine breakdown might have occurred during the few seconds of cooling, but the values obtained in the few seconds immediately after electrical stimulation showed a rapid return to the normal level, indicating that under these conditions resynthesis of acetylcholine was also rapid. Even if anoxia occurred, the brain would contain ample phosphocreatine and adenosine triphosphate to enable resynthesis of acetylcholine to take place. It is therefore possible that resynthesis proceeded during the 4 seconds of cooling, so that the acetylcholine content of the brain during electrical stimulation was actually lower than would appear from the figures obtained. From the slope of the resynthesis curve a rough estimate of the rate of acetylcholine synthesis in the brain *in vivo* could be made: the calculated rate was 7 $\mu\text{g/gm.}$ fresh brain tissue/minute.

The onset of convulsions was associated with a second fall in the acetylcholine content, which fell more gradually to 50 per cent of the normal value in 40 to 50 seconds. After the convulsions were over, the acetylcholine level again began to rise, but at this stage some of the animals were in an excitable state and the return to normality in the brain acetylcholine level was more gradual than immediately after electrical stimulation.

Chromodacryorhesis. It was noted that chromodacryorhesis ('bloody tears') occurred after electrical stimulation by scalp electrodes under the conditions described. Since it occurred only when the stimulation was sufficient to cause coma and not when the shock was insufficient to produce this effect, it should probably be attributed to the central stimulation rather than to a local action. The observation appears relevant since it is known that chromodacryorhesis is produced by injection of a small amount of acetylcholine (2.0 $\mu\text{g/kg.}$) in the rat, and it has been regarded as an indicator of a release of acetylcholine into the general circulation (8).

DISCUSSION

Evidence is presented that the acetylcholine content of the rat brain, determined after rapid fixation by freezing with liquid air, varies with the physiological state of the animal. The level was significantly raised in animals taken during anesthesia and during sleep; it was significantly lowered in animals taken during emotional excitement, after electrical stimulation of the brain and during convulsions. The acetylcholine level thus appeared to vary inversely with the degree of functional activity of the brain. The observed changes were relatively large. Electrical stimulation for 1 to 3 seconds caused a loss of over 50 per cent of the whole brain acetylcholine. The level was raised in anesthesia 40 per cent above the normal value and more than 300 per cent above that of animals taken during convulsions. Studies of the rate of

loss and resynthesis of acetylcholine *in vivo* showed that the changes were rapidly reversible. The rate of resynthesis after electrical stimulation was such that under these conditions the brain could synthesize approximately 7 μ g. acetylcholine per gm., or more than five times the normal brain content, in one minute. The changes were shown by the use of specially rapid methods and the results do not therefore disagree with those of other investigators who have used less rapid techniques.

These observations are clearly relevant to the problem of nervous transmission in the central nervous system. Various hypotheses of acetylcholine action have been discussed elsewhere and different aspects of the subject have been reviewed by Feldberg (9), Eccles (10), Nachmansohn (11) and Dale (12). It is known that the central synapses are highly sensitive to acetylcholine applied to the cerebral cortex, but there has been no direct evidence that acetylcholine is actively concerned in transmission in the central nervous system. The present work gives evidence that functional activity of the brain is associated with a fall in the total acetylcholine content. It gives no evidence of the mechanism involved, but it gives support to the view that nervous activity in the central nervous system involves the liberation and breakdown of acetylcholine.

Besides the changes in the acetylcholine level, stimulation has previously been shown to produce changes in the lactic acid and in other metabolites in the brain (1-3). The present observations on acetylcholine can be related to the other changes if it is assumed that the lactic acid formation corresponds to an increased utilization of carbohydrate required to supply energy for the resynthesis of acetylcholine during increased functional activity of the brain. Experiments *in vitro* suggest that the energy required for acetylcholine synthesis can be supplied through the mediation of the high-energy phosphate esters. In agreement with this view, the brain lactic acid level is low and the phosphocreatine is high during anesthesia, when acetylcholine synthesis may be expected to be minimal, while during electrical stimulation or convulsions the relations are reversed.

Convulsions. Acetylcholine produces spike discharges when applied after eserine to the cerebral cortex, and this has led to the suggestion that it may be specifically concerned in the mechanism of epileptic seizures (13). It was noted in the present experiments on electrically induced convulsions that the convulsions did not start until the acetylcholine had returned, after the initial fall, to the normal value (fig. 1). The convulsions also came to an end with the second fall in the acetylcholine level. This gave evidence that the two may be related and that an adequate acetylcholine level may be one of the requirements for convulsive activity of this kind. Activity would appear to be depressed if the acetylcholine falls to too low a value, as must happen if for any reason the rate of resynthesis cannot keep pace with the rate of acetylcholine liberation and breakdown. This agrees with the recent work of Hyde, Beckett and Gellhorn, who have shown that convulsive activity can be restarted, after it has come to an end, by the direct application of acetylcholine to the cerebral cortex (14). A similar explanation might be given to the increased frequency of seizures and of E.E.G. seizure patterns seen in epileptics during the earlier stages of anesthesia and in sleep, when the acetylcholine level may be expected to be high.

The observed changes in the acetylcholine level offer an explanation for some of the facts, but it is clear that other factors, apart from acetylcholine, would be needed to account for the occurrence of convulsions. The brain is apparently more irritable after electrical stimulation, for convulsions occur then when the acetylcholine level is no higher than normal. This abnormal irritability suggests the additional action of some other toxic factor and it may be relevant that ammonia, which is a powerful cerebral irritant, has been shown to be liberated in significant amounts by the action of convulsant drugs and electrical stimulation on the brain (3). The combined effects of acetylcholine and a toxic factor such as ammonia might offer a more satisfactory basis for interpreting the phenomena of convulsions.

SUMMARY

The acetylcholine content of the rat brain depends on the physiological state. It is increased in sodium pentobarbital anesthesia and in sleep; it is reduced in emotional excitement, in electrical stimulation and in convulsions. It would thus appear to vary inversely with the degree of activity of the brain. The changes are relatively large: the acetylcholine level in anesthesia is 300 per cent above that in convulsions. The fall in the brain acetylcholine in electrical stimulation is a transient and rapidly reversible effect. The rate of resynthesis of acetylcholine in the brain *in vivo* after electrical stimulation is of the order of 7 μ g. acetylcholine per gram per minute under these conditions.

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INFLUENCE OF DESOXYCORTICOSTERONE ACETATE ON LIVER AND MUSCLE GLYCOGEN OF ADRENALECTOMIZED ANIMALS

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IT HAS often been stated that the corticosteroid hormones belong to two groups, one influencing carbohydrate metabolism, the other electrolyte metabolism. The representative of the first group is corticosterone; that of the second, desoxycorticosterone. It is often stated, especially in clinical literature, that only the 11-oxy-corticosteroids have an influence on carbohydrate metabolism (1-4). This has been contradicted by Verzár (5), who has claimed since 1940 that the main difference would not be one of quality but rather of velocity, desoxycorticosterone acting slower.

Grollman (6) writes: "Desoxycorticosterone fails to remedy the defect in carbohydrate metabolism observed in adrenal cortical insufficiency. . . ." And "Desoxycorticosterone and its 17-hydroxy-derivative fail to affect either the carbohydrate metabolism or the working capacity, whereas the corticosterone compounds exert. . . ." Ingle and Kuizenga (7) wrote in 1945, "11-desoxycorticosterone influences the carbohydrate metabolism of adrenalectomized animals in at least two different ways. First, . . . tends to restore normality to the circulatory mechanisms . . . ; second, . . . is weakly active in stimulating the formation of carbohydrate from non-carbohydrate sources. . . ."

Ingle (8) in his review of 1945 noted, "But it has been established beyond reasonable doubt that the compound 11-desoxycorticosterone is deficient in its ability to stimulate the formation of new carbohydrate from non-carbohydrate sources". It was said by Olson *et al.* (9) and often repeated by others that "There is now a wealth of evidence to indicate that neither those steroids which are not ketonic at C₃ and α , β -unsaturated in Ring A nor those which are without an oxygen substituent at C₁₁ have any influence upon carbohydrate and protein metabolism in either the intact or adrenalectomized animal."

In the present paper it is shown that desoxycorticosterone, if given in daily doses which keep adrenalectomized rats in a healthy state, has an action on glycogen formation and keeps normal values of liver and muscle glycogen. Nothing will be said about short-term experiments like those of Ingle (3) and Olson (4) *et al.*, where undoubtedly 11-desoxycorticosterones were inferior in their action, which will be analyzed later.

Three different types of feeding experiments on normal and adrenalectomized animals were used: a) carbohydrate rich, but protein and fat containing diet; b) forced feeding of glucose by stomach tube to starved animals; c) protein diet. Desoxycorticosterone acetate was given i.m. in oily solution.³

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METHODS

Male rats of 70 to 160 gm. were adrenalectomized. Animals of the same age and weight were kept as controls. All were fed with the usual stock diet. Food was given twice daily at 9 A.M. and at 4 P.M. They were weighed before the afternoon feeding and part received i.m. injections of desoxycorticosterone acetate.

The animals were pitthed and then bled from the carotids. One hundred to 300 mg. of liver and 300 to 500 mg. of trunk muscles were quickly weighed on a torsion balance and plotted in centrifuge tubes with 2 cc. boiling 20 per cent NaOH. Two and a half to 3 minutes for liver and 4 to 5 minutes for muscle elapsed from the death of the animals; this time was carefully kept.

The tissues were totally dissolved in the NaOH and then 3 cc. hot concentrated ethyl alcohol (96%) was added and mixed. Glycogen was precipitated in 30 minutes in the ice chest and then centrifuged. The precipitate was dissolved in 1 cc. hot 1 per cent NaCl, again precipitated with 2 cc. concentrated ethyl alcohol and again left for 30 minutes in the ice chest, centrifuged, washed with 2 cc. concentrated ethyl alcohol and then dried on a water-bath. The precipitate of glycogen was hydrolyzed with 4 cc. 2 N H_2SO_4 in boiling water for $1\frac{1}{2}$ hours, then made slightly alkaline with 5 cc. 2 N Na_2CO_3 , transferred in 50 cc. bottles, filled with distilled water and in an aliquot part sugar estimated by Hagedorn-Jensen's method.

Glycogen is given in the tables as grams of glucose per 100 gm. wet tissue weight.

EXPERIMENTAL RESULTS

Animals on Mixed (Stock) Diet

Series I A and B. In the first series, 17 adrenalectomized animals (series I A) on a mixed diet were injected with desoxycorticosterone. On the first 3 days 2 mg., later 1 mg., daily was given i.m. (The first injection was given just after adrenalectomy.) No injection was given on the day of estimation and the food was taken away $1\frac{1}{2}$ hours before. The animals were killed in groups 10, 14, 16, and 20 days after adrenalectomy. With each group an approximately equal number, altogether 17 normal animals, was worked up for liver and muscle glycogen (series I B).

All the adrenalectomized animals were kept alive by desoxycorticosterone acetate. During the injections they increased in weight as did the other animals of the control series. To prove the action of desoxycorticosterone 5 animals of this series were not injected after the 20th day. Their weight curve immediately turned down and with constant loss of weight the animals died after 15 to 17 days. They were typically adynamic before death. Table 1 shows the glycogen content of liver and muscles of these animals (I A), compared with normals. There is no difference between these groups, either in liver or muscle glycogen.

Series II A and B. Fourteen adrenalectomized animals were daily injected with desoxycorticosterone. On the first day after operation they received 2 mg. and on the following days 1 mg. daily. A second group of 15 adrenalectomized animals was not treated with desoxycorticosterone. All these animals were killed in groups 2 to 5 days after adrenalectomy and their liver and muscle glycogen was estimated. Table 1 gives the mean values.

The mean values in table 1 are calculated from the values of the 2nd to 5th day,

from values of 11 untreated and 10 treated adrenalectomized animals. They show clearly the decrease of liver and muscle glycogen without treatment and the total normal values with treatment.

It is clear that after adrenalectomy in the untreated animals liver glycogen drops down, as is well known, in 1 to 5 days to very low values. In desoxycorticosterone-treated animals glycogen is mostly low on the first, but already normal on the 2nd and 5th day, since the first injection was given directly after the adrenalectomy. Desoxycorticosterone had little or no action on the first day of application but a complete action on glycogen later.

TABLE 1. MEAN VALUES OF LIVER AND MUSCLE GLYCOGEN IN NORMALLY FED ANIMALS

SERIES	GROUP	LIVER GLYCOGEN		MUSCLE GLYCOGEN	
		No. of Animals	Per cent	No. of Animals	Per Cent
I A	Adrenalectomized + DCA 10-20 days after adrenalectomy	15	4.8 ± 0.4	17	0.380 ± 0.22
I B	Normal controls	18	4.8 ± 0.3	17	0.415 ± 0.27
II A	Adrenalectomized, untreated, 2-5 days after adrenalectomy	11	1.5 ± 0.4	11	0.340 ± 0.33
II B	Adrenalectomized + DCA 2-5 days after adrenalectomy	10	4.7 ± 0.6	10	0.440 ± 0.21
Differences between		P for liver		P for muscle	
I B and II A		<0.01		0.05-0.1	
I B and II B		0.8-0.9		0.4-0.5	
I B and I A				0.3-0.4	
II A and II B		<0.01		0.01-0.02	

Glucose Force-Fed Animals

In this and the next series the animals, which were kept on the mixed diet, were fasted for 24 hours and then were given by stomach tube 2 cc. 50 per cent glucose. Three hours later the animals were killed and glycogen estimated.

Series III A and B. Six normal animals were compared with 6 adrenalectomized animals on the 28th day after adrenalectomy after daily treatment with desoxycorticosterone in the same way as in the first series. Table 2 shows that in normal animals liver glycogen had recovered to 1.3 per cent, while in adrenalectomized animals it had reached 1.7 per cent. Muscle glycogen was 0.410 per cent in the normals and 0.475 per cent in the adrenalectomized. Thus no difference between the two groups was present.

Series IV A and B. Four adrenalectomized animals were treated from the first day after adrenalectomy with desoxycorticosterone acetate in the same way as above. The animals were fasted 24 hours, and then were given 2 cc. 50 per cent glucose by stomach tube 3 hours before used. They were killed on the 1st or 2nd day. Five more

adrenalectomized animals were untreated and killed together with the former. The result is shown in table 2. Desoxycorticosterone restored the ability to form liver glycogen in the adrenalectomized animals by the first day after adrenalectomy. Muscle glycogen seemed not quite restored on the second day.

Protein-Fed Animals

The possibility has often been suggested that the adrenal cortical hormones influence glucose formation from proteins. In the following experiments we compared normal and adrenalectomized animals on a protein diet, which was prepared in the following way: Lean beef was cooked in water, finely minced through a meat grinder and cooked once again. Twenty gm. was fed daily to each rat but was not totally

TABLE 2. MEAN VALUES OF LIVER AND MUSCLE GLYCOGEN IN 24-HOUR STARVED, GLUCOSE FORCE-FED ANIMALS

SERIES	GROUP	LIVER GLYCOGEN		MUSCLE GLYCOGEN	
		No. of Animals	Per cent	No. of Animals	Per Cent
III A	Normal controls	6	1.3 ± 0.18	6	0.410 ± 0.40
III B	Adrenalectomized + DCA 28 days after adrenalectomy	6	1.7 ± 0.18	6	0.475 ± 0.25
IV A	Adrenalectomized + DCA 1-2 days after adrenalectomy	4	1.8 ± 0.23	4	0.380 ± 0.08
IV B	Adrenalectomized, untreated, 1-2 days after adrenalectomy	5	0.4 ± 0.22	5	0.280 ± 0.07
Differences between		P for liver		P for muscle	
III A and III B		0.1-0.2		0.1-0.2	
IV B and IV A		<0.01		0.7-0.8	

consumed. Vitamins B₁ and B₂ and A and D in the form of cod liver oil were added to the daily dose.

Groups of normal and adrenalectomized animals were killed on the same days, and estimations of glycogen in liver and muscle were made.

Series V A. Seven normal rats (of 150-200 gm.) were fed with the meat diet 20 to 24 days. All had decreased in weight from the beginning of the pure meat feeding by 19 to 41 gm., but were in good health. They showed very poor or no fat reserves. They were killed 20, 22 and 24 days after the feeding began. Their livers had a glycogen content of 3.543 gm. per cent, their muscles 0.514 gm. per cent.

Series V B. Fourteen rats were kept for one week on pure meat diet and then adrenalectomized. They were not treated with hormone and were killed on the 3rd to 9th day after adrenalectomy, when their body weight had declined from the day of adrenalectomy by 4 to 69 gm. and adynamia was present. The liver glycogen was

extremely low. The mean value was 0.081 gm. per cent. Muscle glycogen had also much decreased to 0.140 gm. per cent mean value.

Series V C. Twenty-three adrenalectomized animals, treated i.m. with 2 mg. desoxycorticosterone acetate daily, were used between the 3rd and 13th day after adrenalectomy. Fifteen animals were fed on a pure meat diet 3, 7 and 10 days before the adrenalectomy and then again after it. In 8 animals meat was fed 14 days before the adrenalectomy, and from then on. All animals were treated with 2 mg. desoxycorticosterone daily. The glycogen content of the liver showed a mean value of 0.890 gm. per cent. In 10 of these 23 animals the value was above 1.1 gm. per cent. In 9 cases, however, it was under 0.4 gm. per cent, but three times under 0.2 gm. per cent, as in untreated animals. Their muscle glycogen was 0.480 gm. per cent mean value.

There is no doubt that the majority of desoxycorticosterone-treated animals could produce glycogen from protein since their liver glycogen was much higher than that of untreated animals. In half of the cases it reached about half the value of normals.

Series VI A and VI B. Since, however, no explanation could be given for the low values in about half of the former series, more experiments were done on these lines. The desoxycorticosterone-treated animals had lost considerable weight in this last series. It was thought that they were not in an optimal state of compensation with 2 mg. desoxycorticosterone daily. Further experiments were being made therefore with 3 mg. desoxycorticosterone daily. In this series the adrenalectomized animals did not lose weight.

Eight normal controls (*series VI A*) were always taken together with the adrenalectomized animals, so that they were for the same time interval on the meat diet. Thirteen adrenalectomized animals (*Series VI B*) at the time of adrenalectomy had been on the meat diet for 6 days. They were killed on the 5th (2), 6th (3), 9th (4) and 10th (4) day after adrenalectomy. The normal controls had a liver glycogen mean value of 3.575 per cent and a muscle glycogen of 0.848 per cent. Animals treated daily with 3 mg. DCA had a liver glycogen mean value of 2.277 per cent with muscle glycogen 0.820 per cent. Table 3 shows all the results of the protein feeding experiments.

From these results it seems to be quite certain that desoxycorticosterone-treated animals on 3 mg./day can produce glycogen from protein. It is questionable whether the difference of the glycogen content of the liver between the normal controls of *series VI A* and desoxycorticosterone-treated adrenalectomized animals (*series VI B*), 3.5 per cent and 2.3 per cent respectively, means an insufficient activity of 3 mg. desoxycorticosterone as in animals with 2 mg./day desoxycorticosterone. However muscle glycogen shows no such differences.

It is certain that 2 mg. desoxycorticosterone daily did not replace the adrenal function completely. While glycogen was produced from protein, this was certainly possible only in a much smaller degree. This is a striking difference to the glycogen production on a carbohydrate diet or glucose-forced feeding. Three mg./day however was sufficient for muscle glycogen, and nearly sufficient for liver glycogen production to become normal.

DISCUSSION

Three different types of experimental procedure were used. In the first we kept normal and adrenalectomized rats on a mixed diet, the usual stock diet. Normal adrenalectomized untreated and desoxycorticosterone-treated rats were compared. Liver and muscle glycogen was analyzed when the latter were kept alive for various lengths of time. There was no difference between the liver and muscle glycogen of normal and desoxycorticosterone-treated adrenalectomized rats.

In another type of experiment the rats were kept on the same stock diet, were fasted for 24 hours and then force-fed with 1 gm. glucose; 3 hours later glycogen

TABLE 3. MEAN VALUES OF LIVER AND MUSCLE GLYCOGEN IN PURE PROTEIN-FED ANIMALS

SERIES	GROUP	LIVER GLYCOGEN		MUSCLE GLYCOGEN	
		No. of Animals	Per Cent	No. of Animals	Per Cent
V A	Normal controls	7	3.543 ± 0.46	7	0.514 ± 0.03
V B	Adrenalectomized untreated	14	0.081 ± 0.016	14	0.140 ± 0.018
V C	Adrenalectomized treated with 2 mg. DCA	23	0.890 ± 0.155	23	0.480 ± 0.033
VI A	Normal controls	8	3.575 ± 0.40	8	0.848 ± 0.060
VI B	Adrenalectomized treated with 3 mg. DCA	13	2.277 ± 0.18	13	0.820 ± 0.033
Differences between		P for liver		P for muscle	
V A and V B		<0.01		<0.01	
V A and V C		<0.01		>0.9	
V B and V C		<0.01		<0.01	
VI A and VI B		<0.01		0.6-0.7	

was estimated. Again there was no difference between normal and adrenalectomized, desoxycorticosterone-treated animals, while untreated animals had very low values.

The third type of experiment was done on animals on a meat (i.e., practically pure protein) diet. It was noticed that in these experiments 3 mg. of desoxycorticosterone had to be given daily to keep the animals in such a normal state that liver glycogen was nearly and muscle glycogen quite equal to that of the normals. It seems possible that the usual doses of desoxycorticosterone to keep adrenalectomized animals alive on a stock diet are not equal to those which are needed for animals on a pure meat (protein) diet. There seems to be reason to believe that especially high hormone doses are needed for the latter.

Thus desoxycorticosterone restores glycogen production to normal from carbohydrate or protein in liver and muscles of adrenalectomized animals which are kept alive for some time.

All of these experiments were made on animals which were treated for 2 to 28 days with desoxycorticosterone. Nothing is said about the possibility of influencing glycogen production in a shorter period. The reason for these experiments was to show that desoxycorticosterone has a strong glycogenetic action if given continually. It brings liver and muscle glycogen to normal.

SUMMARY

Desoxycorticosterone-treated rats are able to restore liver and muscle glycogen after several days to normal values. This was found to be true with rats on a mixed diet, or starved and then glucose force-fed, or on a protein diet.

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COMPARISON BETWEEN GLYCOGENETIC PROPERTY OF DESOXYCORTICOSTERONE, 11-DEHYDRO-17-HYDROXY-CORTICOSTERONE (COMPOUND E) AND ADRENAL CORTICAL EXTRACT

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IN THE preceding paper (1) it was shown that desoxycorticosterone acetate (DCA) has a glycogenetic effect in animals, which are kept alive with this cortical steroid. This paper demonstrates the difference between this formerly so called 'salthormone' and a typical 'carbohydrate hormone,' 11-dehydro-17-hydroxycorticosterone (Compound E), which has recently been available to us in large enough quantities.² Analytical methods were the same as in the former paper (1). All animals were kept on the pure protein diet described there, consisting of cooked meat with the necessary vitamins added. Male rats of 130 to 180 gm. were used.

EXPERIMENTS

Experiments with Compound E and DCA. Series I was done on 33 rats (table 1). The animals were adrenalectomized after 13 to 14 days on the protein diet. Adrenalectomized animals, if they survived 4 to 7 days without treatment, had extremely low liver and muscle glycogen values. If they were treated for 7 to 14 days after the adrenalectomy with 2 mg. DCA or 2 mg. Compound E daily, when all animals were healthy and lively, liver glycogen was 2.26 gm. per cent in the DCA and 3.57 gm. per cent in the E group. There was no difference in muscle glycogen.

Normal animals were killed together with the former. They were on the protein diet the same length of time. The liver glycogen was 1.97 gm. per cent and the muscle glycogen 0.49 gm. per cent.

In *series II* we used 25 animals (table 2). They were kept on the protein diet 3 days before adrenalectomy. The normal animals were kept as long on this diet as the adrenalectomized. They had a liver glycogen value of 1.9 gm. per cent.

After adrenalectomy and a daily treatment of 4 mg. DCA after 5 to 16 days, we found 1.2 gm. per cent liver glycogen. However, only 1 mg/day of Compound E gave 1.9 gm. per cent liver glycogen.

All our data on animals on protein diet after prolonged treatment have been collected in table 3.

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² We wish to thank Ciba A. G., Basle, for the desoxycorticosterone-acetate; Merck & Co., Rahway, for Compound E and Upjohn Co., Kalamazoo, for the adrenal cortical extract, for these experiments.

From these experiments on 77 adrenalectomized animals kept alive for 5 to 16 days after the adrenalectomy with corticosteroids, it seems that while DCA can produce normal liver and muscle glycogen values in doses of 2 to 3 mg/day, Compound E is somewhat superior to it. Thus in *series I* 2 mg/day DCA produced 2.2 and Compound E 3.6 per cent liver glycogen. In *series II* 1 mg. Compound E daily produced more liver glycogen than 4 mg. DCA (but 4 mg. DCA did not produce more than 2 mg. DCA in the other series). Muscle glycogen showed no difference.

TABLE 1. GLYCOGEN CONTENT OF LIVER AND MUSCLE, PER CENT WET WEIGHT

DAYS AFTER ADRENAL- ECTOMY	'E' TREATED, 2 MG/DAY		DCA TREATED, 2 MG/DAY		UNTREATED		NORMAL CONTROLS	
	Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle
4					0.10	0.16		
5					0.10	0.18		
6					0.10	0.15		
7	1.90	0.50	3.90	0.77	0.10	0.15	3.00	0.59
	1.60	0.65	4.30	0.65	0.10	0.12		
9	6.80	0.85	1.10	0.66			1.10	.55
	3.40	0.68	0.75	0.45				
	4.20	0.60						
11	3.50	0.36	0.30	0.21			1.90	0.26
	3.50	0.48	1.30	0.36				
	4.10	0.44						
12	3.50	0.59	4.30	0.79			3.00	0.45
	3.70	0.54	1.70	0.67				
13			2.30	0.60				
14	3.10	0.59	2.60	0.61			1.90	0.58
							1.10	0.48
							1.80	0.54
Mean values	3.57±0.16	0.57±0.04	2.26±0.47	0.58±0.06	0.10 ¹	0.15±0.00	1.97±0.30	0.49±0.26
No. of animals	11		10		5		7	

¹ ±0.05 as limit of error of estimation.

Experiments with Adrenal Cortical Extract (Upjohn). In *series III*, 10 protein-fed animals were treated after adrenalectomy with 0.5 cc. extract injected daily i.m. Three animals died. Seven surviving animals showed between the 7th and 11th day the following very low mean values: liver glycogen 0.15 gm. per cent, muscle glycogen 0.26 gm. per cent.

In *series IV*, 6 protein-fed animals were adrenalectomized after 4 days on the diet. They were then treated daily with 2 cc. extract i.m. for 10 days. The extract was injected once daily. Glycogen analyses were made 24 hours after the last injection. Parallel animals were untreated, or treated with 2 mg. DCA/day. The results are shown in table 4.

TABLE 2. GLYCOGEN CONTENT OF LIVER AND MUSCLE, PER CENT WET WEIGHT

DAYS AFTER ADRENALECTOMY	E ¹ TREATED, 1 MG/DAY		DCA TREATED, 4 MG/DAY		NORMAL CONTROLS	
	Liver	Muscle	Liver	Muscle	Liver	Muscle
5	1.40	0.58	0.44	0.41	2.20	0.66
	1.20	0.37	0.30	0.40		
7	2.70	0.43	1.00	0.55	1.60	0.50
	3.10	0.41	2.70	0.47		
13	2.90	0.63	0.20	0.50	0.79	0.52
	0.97	0.43	0.10	0.23		
14	1.00	0.56	0.55	0.82	2.30	0.58
	0.73	0.64	1.50	0.80		
16	2.50	0.38	2.80	0.71	2.70	0.41
	2.10	0.49	2.30	0.70		
Mean values	1.90±0.28	0.49±0.03	1.20±0.33	0.56±0.06	1.90±0.34	0.53±0.04
No. of animals	10		10		5	

TABLE 3. GLYCOGEN CONTENT OF LIVER AND MUSCLE, PER CENT WET WEIGHT

SERIES	TREATMENT	DAYS	GLYCOGEN CONTENT		NO. OF ANIMALS
			Liver	Muscle	
		<i>maxim.</i>	<i>gm. %</i>		
V C ¹	DCA	13	0.89±0.155	0.48±0.033	23
	2 mg/day				
I	2	14	2.26±0.47	0.58±0.06	10
VI B ¹	3	13	2.28±0.018	0.82±0.033	13
II	4	16	1.20±0.33	0.56±0.06	10
II	E 1	16	1.90±0.28	0.49±0.03	10
I	2	14	3.57±0.16	0.57±0.04	11

¹ See the former paper (1).

TABLE 4. GLYCOGEN CONTENT OF LIVER AND MUSCLE, PER CENT WET WEIGHT

DAYS AFTER ADRENALECTOMY	TREATMENT	GLYCOGEN CONTENT	
		Liver	Muscle
		<i>gm. %</i>	
10	Untreated	0.15	0.49
10	Untreated	0.10	0.20
10	2 cc. untreated extract	0.18	0.63
10	2 cc. untreated extract	0.16	0.56
10	2 mg. DCA	1.30	0.76
10	2 mg. DCA	1.30	0.78

Thus we were unable to keep the glycogen content of the liver even fairly normal with 0.5, or 2.0 cc. extract/day, while in parallel experiments DCA gave the usual normal (or nearly normal) glycogen values in the liver. In muscles 2 cc. of the extract gave relatively high glycogen values, which, however, still were lower than with desoxycorticosterone.

Short-time Experiments. Since it is known from the experiments of Reinecke and Kendall (2), Olsen (3) and Ingle (4) that in experiments of a few hours' duration the

TABLE 5. GLYCOGEN CONTENT OF LIVER AND MUSCLE, PER CENT WET WEIGHT

HOURS AFTER INJECTION	DCA 2 MG. GLYCOGEN CONTENT		'E' 2 MG. GLYCOGEN CONTENT		EXTRACT 3 CC. GLYCOGEN CONTENT	
	Liver	Muscle	Liver	Muscle	Liver	Muscle
	gm. %		gm. %		gm. %	
6	0.68	0.56	1.80	0.86	0.14	0.37
	0.10	0.48	1.70	0.64	0.11	0.25
	0.10	0.36				
	0.20	0.38				
	0.12	0.41				
12	0.80	0.43	0.74	0.52	1.90	0.39
	1.40	0.75	3.60	0.85	0.66	0.23
	1.50	0.41	4.90	0.75		
	0.84	0.25				
24	0.75	0.95	4.10	0.87	0.20	0.32
	0.20	0.41	4.50	0.87	0.34	0.37
	0.41	0.38	6.00	0.83		
	0.50	0.65	1.90	0.55		
	0.64	0.43				
	1.60	0.59				
	1.20	0.66				
48	0.10	0.41	0.55	0.60		
	0.12	0.66	1.10	0.64		
72	0.10	0.39	0.30	0.45		
	0.30	0.46	0.50	0.47		
			0.52	0.43		
			2.00	0.39		

so-called 'carbohydrate hormones' show much higher glycogenetic capacity, we made experiments of much shorter duration.

The animals were fed one week on the protein diet, then adrenalectomized and kept alive with daily injections of 2 mg/day DCA, during 6 days. On the 7th day they did not receive DCA. Before they were fed on the 8th day at 9 A.M. they were injected with either DCA (2 mg.), Compound E (2 mg.) or cortical extract (3 cc.). Some animals were killed after 6 hours, other at 12, 24, 48, or 72 hours later. The results of 41 experiments are shown in table 5.

DISCUSSION

From the above it can be confirmed that Compound E (11-dehydro-17-hydroxycorticosterone) has a definitely stronger action on carbohydrate metabolism in protein-fed animals than desoxycorticosterone in the short experiments. Desoxycorticosterone had no effect after 6 hours, while Compound E by this time gave high liver and muscle glycogen values. After 12 to 24 hours with DCA, 1.2 to 1.6 gm. per cent liver glycogen was reached, while with Compound E values more than three times as high (3.6-6.0 gm. per cent glycogen) were seen. After 48 hours the DCA

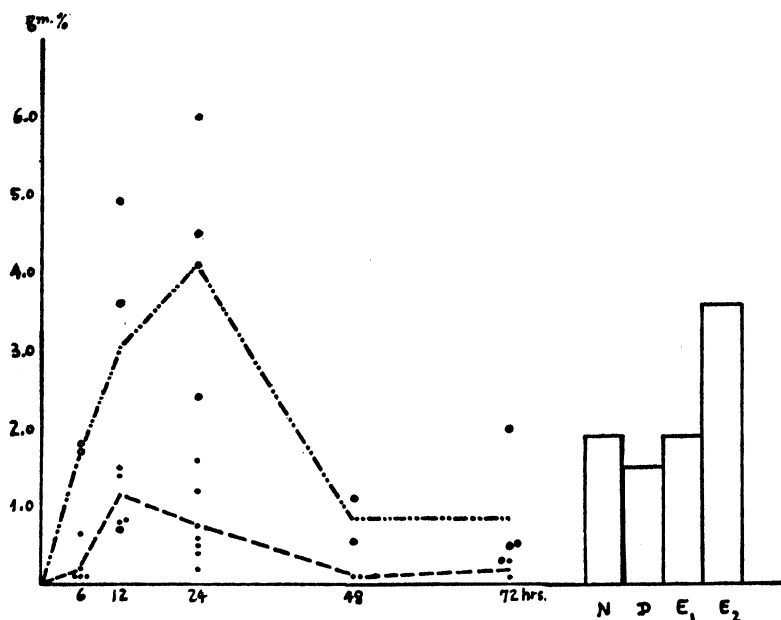


Fig. 1. LIVER-GLYCOGEN after single injection and after continuous treatment with DCA or Compound E. In the left part the liver glycogen content at the intervals of 6, 12, 24, 48 and 72 hours after a single injection of Compound E or DCA is plotted (table 5). In the right part the columns give liver glycogen middle values of DCA and E in long-term experiments of 3 to 16 days' duration from all experiments of table 3 and normal controls from table 1 and 2 together. — — — DCA 2 mg. single injection. — · — · —, E 2 mg. single injection. N, normal controls. D, DCA 2 to 4 mg/day during 3 to 16 days; E₁, Compound E 1 mg/day during 3 to 16 days. E₂, Compound E 2 mg/day during 3 to 16 days.

effect had totally disappeared, but with Compound E some small activity was still recognizable.

The difference between DCA and Compound E was, however, smaller in experiments with daily treatment over a longer period of 7 to 15 days. Then desoxycorticosterone-treated animals also showed high values of liver and muscle glycogen. The number of animals which we treated was not large enough to give a more quantitative comparison in such experiments of long duration. However, a relation of 1 to 2 in favor of Compound E is probable. The mean value of the DCA-treated animals reached the glycogen content of normal protein-fed rats.

Thus the action of Compound E is especially quick and strong compared with DCA if used in single doses. It is much less different in chronic experiments. It has been supposed for several years (5, 6) that the difference between the action of the two groups of steroids with regard to the carbohydrate metabolism is not qualitative but mainly quantitative. Compound E has a quick and strong action on glycogen formation. DCA acts much slower and therefore less after a single injection. But it brings glycogen production to about normal values if given in daily repeated doses (see fig. 1). We have not studied the relation of these two steroids in animals on a carbohydrate diet as yet.³

Adrenal cortical extract in quantities of 2 cc/day, injected intramuscularly, showed in chronic experiments of 10 days' duration and short-time experiments of 6, 12 or 24 hours' duration, less activity than 2 mg. DCA. We had not enough material to test greater doses.

It is shown, however, in the former and present papers, that especially in long-term experiments the difference in glycogen production between a so-called 'electrolyte hormone' (as DCA) and a 'carbohydrate hormone' (as Compound E) is not a qualitative one, but a quantitative one only.

SUMMARY

The action of 11-dehydro-17-hydroxycorticosterone (Compound E), desoxycorticosterone (DCA) and adrenal cortical extract upon glycogen formation in liver and muscle of adrenalectomized rats on a pure protein diet has been tested. Compound E has a much stronger and more rapid effect than DCA in short-time experiments. The difference is smaller in experiments with long periods of treatment, where desoxycorticosterone also brings the liver glycogen values of adrenalectomized rats to near normal.

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INFLUENCE OF ESTROGENS ON THE ACUTE X-IRRADIATION SYNDROME

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IT HAS been reported that estradiol benzoate, administered in a single dose 9 to 10 days prior to total-body x-irradiation, increases the resistance of male mice to the x-rays (1-3). On the other hand, injection of the estrogen on the day of irradiation appears to potentiate toxicity. The mechanism whereby pretreatment with an estrogen can alter the toxic effects of x-rays is unknown. Although many phases of the biologic response to ionizing radiations have been well defined experimentally, the factors contributing to radiosensitivity and the basis of radiation death still remain largely a matter for conjecture. It seemed important, therefore, to investigate further this interesting phenomenon of estrogen protection against x-rays.

METHODS

Male and female CF₁ mice, weighing 20 to 25 grams and maintained on a diet of Derwood Checkers and water *ad libitum*, were used in these studies. Animals of the same sex were used in individual experiments. For the irradiation, mice were placed in individual cellulose acetate exposure cells and were given a total-body exposure in groups of 16. Equal numbers of controls and experimentals were irradiated in each exposure. The radiation factors were 200 kv., 15 ma., 0.5 mm. Cu and 3.0 mm. Bakelite filters, target distance 10.8 cm., dose rate 20 r per minute. Male mice received 500 r, females 550 r (measured in air). Several types of experiments were performed. These are considered under the following headings.

Influence of Time of Injection of Estradiol Benzoate on Survival after X-Irradiation. Estradiol benzoate (0.166 mg. in 0.1 cc. sesame oil per mouse I.M.) was given in a single injection at either 35, 25, 15, 10, 5, or 2 days before irradiation, or at the time of exposure or 2 days afterward. Irradiated controls for the above groups received injections of sesame oil (0.1 cc. I.M.) at comparable times.

Effect of Estradiol Benzoate on Peripheral Blood Count and on Organ Weights. Blood sampling was accomplished by making a deep cut in the tail with a sharp razor blade and using a free flow of blood. Determinations on the peripheral blood included: total leucocyte count, erythrocyte count, hemoglobin and a differential leucocyte count made on dry smears prepared with Wright's stain. After the blood sample was obtained, the mice were weighed and killed with sodium pentobarbital. The adrenals, spleen, thymus, inguinal lymph nodes and kidneys were removed, dissected free of fat and weighed on a torsion balance. Certain tissues were then prepared for microscopic examination. The histologic observations have not been completed. Animals in the different groups which were to be bled and killed at various intervals were distributed in random fashion among the different cages and levels of the mouse rack.

It was felt that this sampling procedure for obtaining the hematologic data would be superior to the frequent repeated bleeding of mice which would otherwise be necessary. Irradiated and nonirradiated mice pretreated with a single injection of estradiol benzoate were compared with similar mice receiving sesame oil.

Importance of Adrenals for Protective Action of Estradiol Benzoate. Mice were bilaterally adrenalectomized under sodium pentobarbital anesthesia 3 to 7 days prior to injection of estradiol benzoate or sesame oil. Part of the animals were irradiated 10 days after injection of the latter materials, the remainder served as nonirradiated controls. All of the adrenalectomized mice were given 1 per cent sodium chloride in the drinking water. In addition, some of the mice received daily subcutaneous injections of 0.2 cc. aqueous adrenal cortical extract (Wilson).

TABLE 1. EFFECT UPON SURVIVAL RATES OF ALTERING THE TIME OF INJECTION OF ALPHA-ESTRADIOL BENZOATE (0.166 MG/MOUSE I.M.) RELATIVE TO X-IRRADIATION (500 R) OF CFI MALE MICE

TIME OF INJECTION RELATIVE TO X- IRRADIATION, DAYS	ESTRADIOL					SESAME OIL				
	% survival, weeks after irradiation					% survival, weeks after irradiation				
	Total no. of mice	1	2	3	4 ¹	Total no. of mice	1	2	3	4
2	32	78	9	0	0					
0	54	83	20	15	13	39	95	46	31	28
-2	32	97	36	22	22					
-5	54	94	67	63	61	18	95	61	33	33
-10	126	88	80	80	79	211	91	39	28	28
-15	29	93	81	69	69	30	77	47	44	44
-25	26	85	45	42	42	25	96	32	32	32
-35	21	85	28	19	19	29	89	14	7	7

¹ Bold face figures are significantly different ($P < .05$) from the 4-week mean survival of the combined sesame oil controls (28% of 352 mice).

Specificity of the Estrogen Response. Benzestrol (0.5-1 mg/mouse I.M.), progesterone (0.1-0.2 mg. I.M.) alone or in combination with estradiol benzoate, and testosterone propionate (0.1-1.0 mg. I.M.) were given to mice 10 days before x-irradiation. Survival rates were determined as a measure of sensitivity to the radiation. Estradiol benzoate was also administered to mice 10 days before injection of a nitrogen mustard—methyl (2, 2'-dichloro) diethyl amine HCl, 3.5 μ g/gm. subcutaneously—and toxicity compared with that of untreated controls.

RESULTS

Time of Injection of Estradiol Benzoate. Estradiol benzoate exerts its maximal protective influence when it is given 10 days before x-irradiation (28-day survival, 79% estrogen treated; 28% control irradiated, $P < .001$). Estrogen injection at 5 and 15 days before exposure is also effective while pretreatment at 2, 25 or 35 days is apparently without influence on radiosensitivity. When estradiol is administered at the time of irradiation or 2 days after the exposure, toxicity to x-rays appears to

be enhanced, both in terms of absolute survival and of survival time. These findings are summarized in table 1.

Peripheral Blood Changes. The hematologic picture, following the single injection of estradiol benzoate, is characterized by a decrease in both the heterophils¹ and lymphocytes (figs. 1-3). Maximal depression occurs around 10 to 14 days after the injection. The heterophil change ($p < .05$) is more marked and appears to be somewhat more consistent than the lymphocyte response which is of borderline statistical significance. The erythrocyte count is apparently not influenced appreciably by the estrogen (fig. 4).

Although the total leucocyte count is decreased to the same level in both the estrogen pretreated and sesame oil control groups after their x-irradiation, there is a

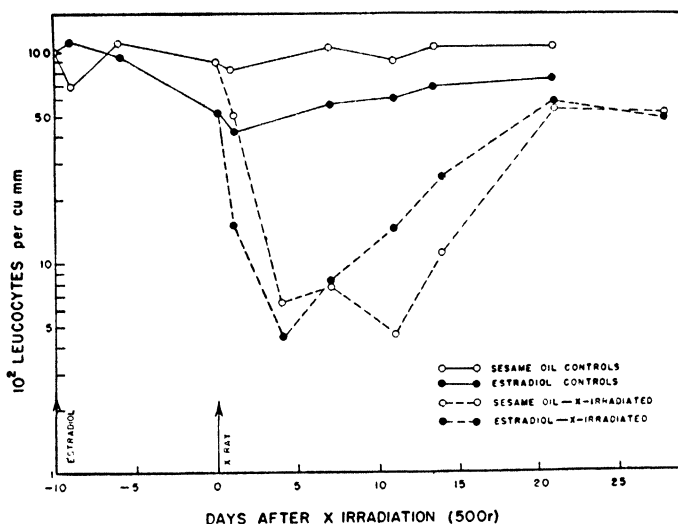


Fig. 1. EFFECT ON TOTAL LEUCOCYTE COUNT of a single injection of estrogen 10 days before x-irradiation with 500 r. Each point represents the average count of 10 to 14 CF₁ male mice.

rather consistent difference in the time course of the response, maximal depression and recovery occurring earlier in the former (fig. 1). The more rapid recovery of leucocytes in the irradiated mice which received a prior injection of estrogen appears to reside in the heterophil component (figs. 2 and 3). This difference in heterophil recovery in the estrogen and control irradiated groups is significant at the 5 per cent level. Erythrocytes in the estrogen pretreated irradiated group are decreased by about 25 per cent 7 days after the exposure but normal values are seen at 11 and 14 days when the red count of the irradiated controls is reduced by 45 per cent (fig. 4). Similar changes are seen in blood hemoglobin.

Organ Weights. Estradiol has no appreciable effect on the weight of the spleen, inguinal nodes or kidney but does increase adrenal weight and accelerate thymic in-

¹ The term heterophil is used to designate the polymorphonuclear leucocyte homologous to the neutrophil in human blood. In the mouse the heterophil is stained with acid dyes.

volution (figs. 5 and 6). Adrenal weight is elevated by 35 to 40 per cent 4 days after estrogen administration and then slowly declines, reaching the control level around 35 days after injection. Radiation-induced involution of the spleen, thymus, and inguinal nodes is not altered by pretreatment with estradiol. However, a somewhat greater increase in adrenal weight is seen in the estrogen-treated irradiated mice.

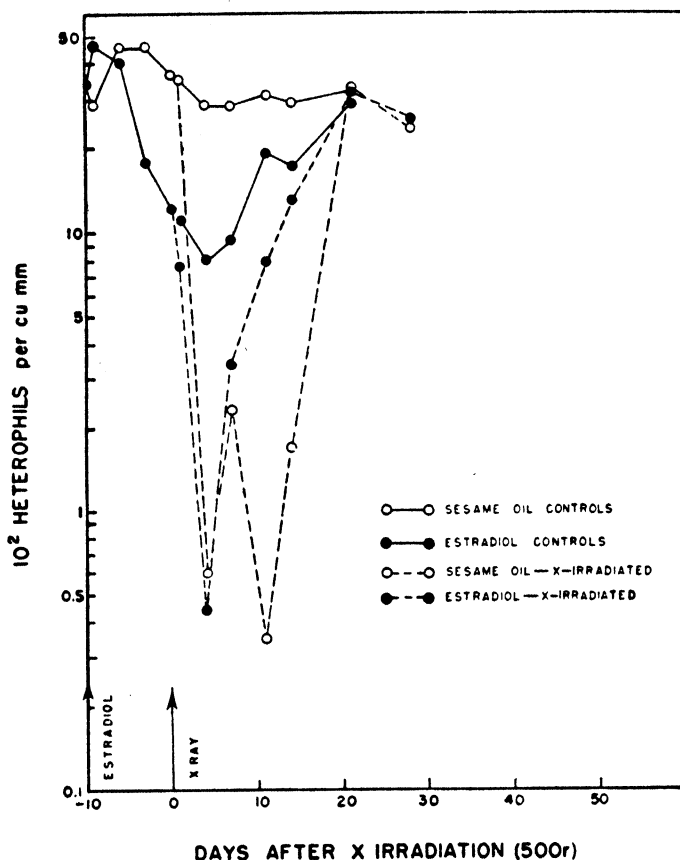


Fig. 2. EFFECT ON HETEROPHIL COUNT of a single injection of estrogen 10 days before x-irradiation with 500 r. Each point represents the average count of 10 to 14 CF₁ male mice.

Importance of Adrenals. Adrenalectomized mice given 1 per cent sodium chloride in the drinking water show a decreased survival time after x-irradiation, although final toxicity remains unchanged. When such operated mice receive a daily adrenal cortical supplement in addition to sodium chloride, they exhibit a sensitivity to radiation which is identical with that of the unoperated controls. Estradiol benzoate administered to adrenalectomized mice maintained only with sodium chloride exerts some protective influence, but this is considerably less than that observed in the operated animals receiving an adrenal cortical supplement (table 2). Estradiol protection in the latter is equivalent to that seen in mice with adrenals (fig. 7).

Specificity of Estrogen Response. Preliminary experiments reveal that benzestrol, a synthetic estrogen, is effective in protecting male and female mice against x-irradiation (table 3). Progesterone and testosterone, on the other hand, appear to be in-

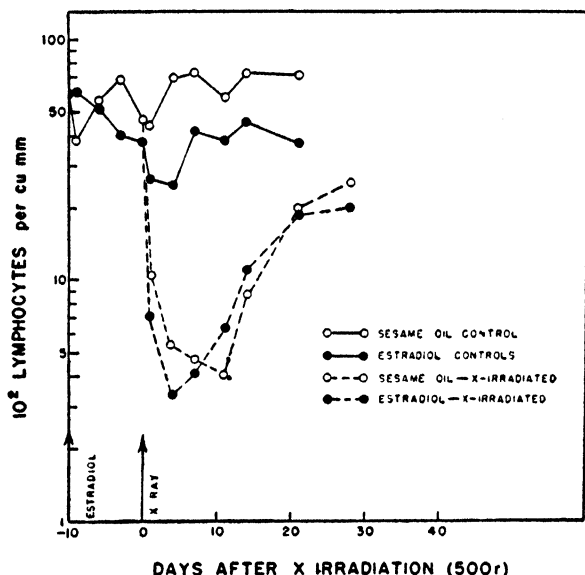


Fig. 3. EFFECT ON LYMPHOCYTE COUNT of a single injection of estrogen 10 days before x-irradiation with 500 r. Each point represents the average count of 10 to 14 CF₁ male mice.

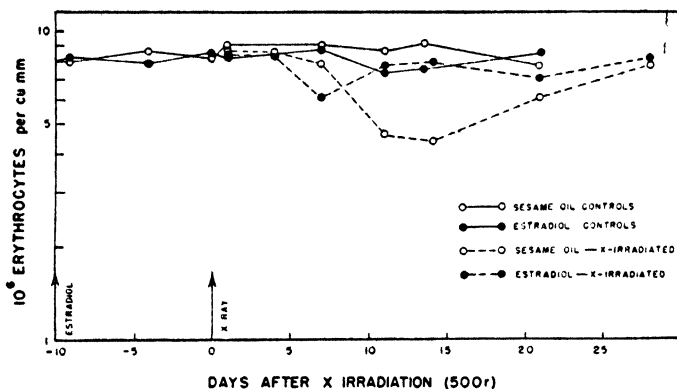


Fig. 4. EFFECT ON ERYTHROCYTE COUNT of a single injection of estrogen 10 days before x-irradiation with 500 r. Each point represents the average count of 10 to 14 CF₁ male mice.

effectual. Combining progesterone with estradiol does not alter the ameliorating influence of the estrogen. These observations are summarized in table 4. Administration of estradiol to mice 10 days before poisoning with a nitrogen mustard does not influence toxicity (table 5).

DISCUSSION

Our findings confirm the earlier observation of Treadwell, Gardner and Lawrence (1) that pretreatment with estradiol benzoate improves the survival of male mice after lethal x-irradiation. It has been demonstrated further that the protective effect of estrogens occurs in female mice as well and that Benzestrol, a synthetic estrogen, but not progesterone and testosterone, is also effective. The time of injection of estro-

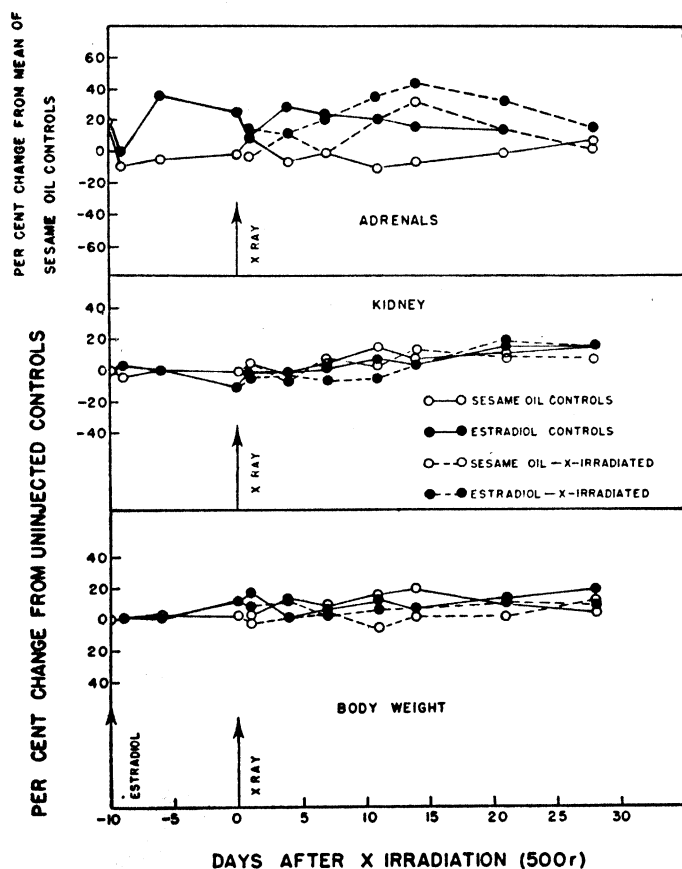


Fig. 5. EFFECT ON ORGAN WEIGHTS of estrogen administration 10 days before x-irradiation with 500 r. Each point represents the average of values obtained on 5 to 7 CF₁ male mice.

gen relative to the time of irradiation appears to be critical, for estradiol is most effective when it is given 10 days before the exposure. Estrogen injection 5 or 15 days before irradiation is also protective but administration at other intervals fails to increase survival and, indeed, may even potentiate toxicity. Although x-irradiation and nitrogen mustard intoxication are rather similar in many respects, estradiol, in dosage sufficient to protect against x-rays, does not influence survival after poisoning with a nitrogen mustard.

Since it is known that thymic involution and adrenal enlargement may occur

after estrogen administration (4, 5), the possibility was first considered that estradiol, perhaps through the intermediary of the adrenal cortex, rendered lymphoid tissue more resistant to irradiation. However, the hematologic data which we have obtained reveal that the lymphocyte count, although depressed by the estrogen, responds to irradiation identically in the estrogen-treated and control-irradiated animals. More-

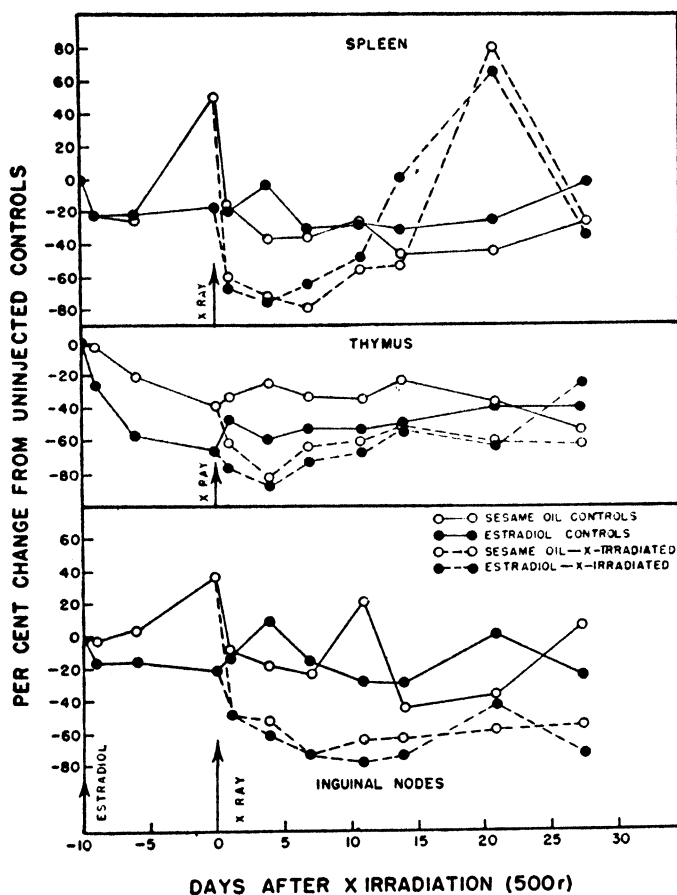


Fig. 6. EFFECT ON ORGAN WEIGHTS of estrogen administration 10 days before x-irradiation, with 500 r. Each point represents the average of values obtained on 5 to 7 CF₁ male mice.

over, lymphoid tissues in the two groups exhibit the same involution after irradiation. Presence of the adrenals is clearly not essential for the estrogen protective action. It is of interest that significantly greater protection is obtained in adrenalectomized animals receiving an adrenal cortical supplement in addition to sodium chloride than in animals receiving only sodium chloride in their drinking water. On the basis of these experiments, it would appear that a certain level of adrenal cortical steroids is necessary, either directly or indirectly, for the maximal estrogen effect. It should be noted that the estrogen per se is more toxic to adrenalectomized animals main-

tained only with sodium chloride than is sesame oil. Twenty-one of 60 adrenalectomized salt-maintained mice succumbed within 10 days of estrogen injection as compared to 8 of 60 similar mice injected with sesame oil. In addition, the operated mice

TABLE 2. INFLUENCE OF ALPHA-ESTRADIOL BENZOATE ON SURVIVAL OF ADRENALECTOMIZED IRRADIATED CF1 MICE¹

EXPERIMENT NO.	GROUP	TREATMENT	SEX	RADIATION DOSE, r	NO. MICE	PER CENT SURVIVAL—WEEKS AFTER X-IRRADIATION			
						1	2	3	4 ²
1	Intact	Sesame oil	M	500	66	83	35	24	24
	Intact	Estradiol	M	500	22	86	82	82	82
	Adrenalectomized	1% NaCl, ACE ³	M		18	95	95	89	82
	Adrenalectomized	1% NaCl, ACE ³ sesame oil	M	500	44	83	27	23	23
	Adrenalectomized	1% NaCl, ACE ³ estradiol	M	500	50	86	78	75	70
2	Intact	Sesame oil	F	550	30	97	23	20	20
	Intact	Estradiol	F	550	27	96	85	81	81
	Adrenalectomized	1% NaCl, sesame oil	F		19	100	95	90	79
	Adrenalectomized	1% NaCl, estradiol	F		16	88	88	75	75
	Adrenalectomized	1% NaCl, sesame oil	F	550	33	51	24	21	18
	Adrenalectomized	1% NaCl, estradiol	F	550	23	70	43	43	39
	Adrenalectomized	1% NaCl, estradiol	F	550	23	70	43	43	39

¹ Estradiol benzoate (0.166 mg. I.M.) injected 10 days before x-irradiation. Controls received equivalent volume of sesame oil. ² Bold face figures are significantly different ($P < .05$) from the 4-week survival of the appropriate irradiated controls. ³ Adrenal cortical extract (Wilson) 0.2 cc. subcutaneously daily.

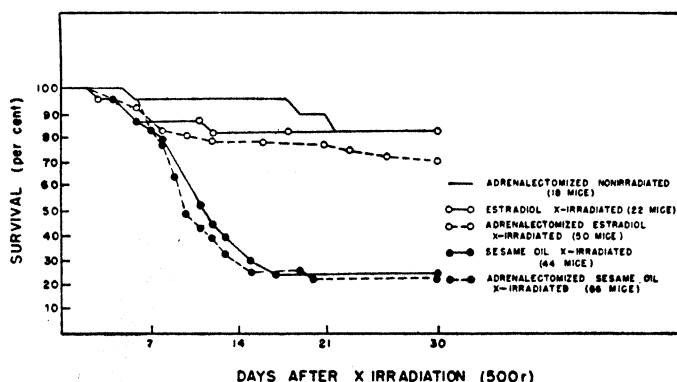


Fig. 7. EFFECT OF ADRENALECTOMY on the protective action of estradiol against x-irradiation of CF1 male mice.

without adrenal cortical supplements appear to be somewhat more sensitive to irradiation than the intact animals.

Significantly, the maximal estrogen protective effect is observed when mice are irradiated during their leukopenic period. This relationship, which is presented graphically in figure 8, along with the observation of a more rapid recovery of the heterophils

in irradiated mice pretreated with estradiol, suggests the possibility that estrogen stimulation renders myelopoietic tissues more resistant to x-rays, perhaps because

TABLE 3. EFFECT OF PRETREATMENT WITH BENZESTROL—2,4-DI (PARAHYDROXYPHENYL) 3-ETHYL HEXANE—ON SURVIVAL OF CFI MICE AFTER X-IRRADIATION

EXPERIMENT NO.	TREATMENT ¹	SEX	RADIATION DOSE, r	NO. MICE	PER CENT SURVIVAL—WEEKS AFTER IRRADIATION			
					1	2	3	4 ²
1	Controls	F	550	20	90	30	25	25
	Benzestrol, 0.1 mg.	F	550	20	95	80	70	70
	Benzestrol, 0.2 mg.	F	550	20	100	85	85	85
2	Controls	M	550	19	100	5	0	0
	Benzestrol, 0.2 mg.	M	550	15	93	20	13	13
3	Controls	M	500	18	100	44	17	17
	Benzestrol, 0.5 mg.	M	500	20	85	65	55	55
	Benzestrol, 1.0 mg.	M	500	20	95	85	85	85

¹ All injections intramuscularly 10 days before x-irradiation. Controls received an equivalent volume of sesame oil. ² Bold face figures are significantly different ($P < .05$) from the 4-week survival of the appropriate irradiated controls.

TABLE 4. EFFECT OF PRETREATMENT WITH ALPHA ESTRADIOL BENZOATE, PROGESTERONE AND TESTOSTERONE PROPIONATE ON SURVIVAL OF MALE CFI MICE AFTER X-IRRADIATION (500 r)

EXPERIMENT NO.	TREATMENT ¹	NO. MICE	PER CENT SURVIVAL—WEEKS AFTER IRRADIATION			
			1	2	3	4 ²
1	Controls, 0.1 cc. sesame oil	41	97	44	32	32
	Estradiol, 0.166 mg.	40	93	85	85	85
2	Controls, 0.1 cc. sesame oil	18	78	33	28	28
	Progesterone, 0.1 mg.	18	83	50	50	50
	Progesterone, 0.2 mg.	18	89	61	55	55
	Progesterone, 0.2 mg. + Estradiol, 0.166 mg.	18	100	100	100	100
	Estradiol, 0.166 mg.	18	89	89	89	89
3	Controls, 0.1 cc. sesame oil	29	87	76	72	72
	Testosterone, 0.1 mg.	30	100	79	66	66
	Testosterone, 1.0 mg.	30	100	59	56	56

¹ All injections I.M. 10 days before x-irradiation. ² Bold face figures are significantly different ($P < .05$) from the 4-week survival of the appropriate irradiated controls.

these tissues are in a proliferative phase during or shortly after the irradiation. This point is receiving further consideration in current studies in which the histologic appearance of myeloid tissues and the cell population of bone marrow smears are being investigated.

Brues (6) has reported that similar lymphopenias are observed in two species (rabbit and guinea pig) with widely different sensitivities following x-irradiation with 200 r. The heterophils, on the other hand, are decreased markedly only in the guinea pig with this dose (LD_{50}). As the radiation dose is increased, a marked depression in heterophils appears in the rabbit as its lethal dose is approached. Brecher *et al.* (7) have observed that lymphocytes and heterophils are decreased rather equally in mice

TABLE 5. EFFECT OF PRETREATMENT WITH ALPHA ESTRADIOL BENZOATE ON SURVIVAL OF MALE CFI MICE AFTER POISONING WITH NITROGEN MUSTARD—METHYL (2,2'-DICHLORO) DIETHYL AMINE HYDROCHLORIDE—3.5 μ G/GM. SUBCUTANEOUSLY

TREATMENT ¹	NO. MICE	PER CENT SURVIVAL—DAYS AFTER NITROGEN MUSTARD					
		3	4	5	6	8	15
Controls, 0.1 cc sesame oil	31	100	64	29	16	16	16
Estradiol, 0.083 mg.	28	100	57	32	11	11	11
Estradiol, 0.166 mg.	23	91	65	17	9	4	0

¹ All injections intramuscularly 10 days before nitrogen mustard poisoning.

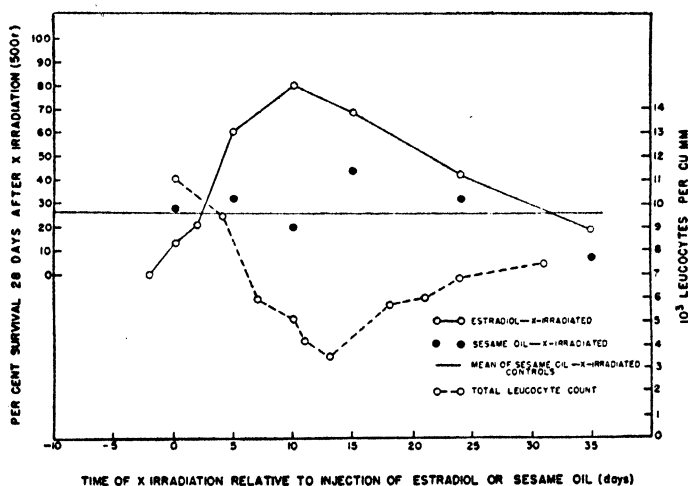


Fig. 8. RELATIONSHIP OF THE ESTROGEN protective effect to the change in total leucocyte count induced by a single injection of estradiol benzoate (EDB).

after an apparently non-lethal exposure to x-rays (400 r). However, their data reveal that there is a fairly rapid recovery of the heterophils at this dose. That there is some correlation between heterophil response and lethal effect is also indicated in our finding that a rapid recovery of heterophils but not of lymphocytes occurs in mice treated with estradiol 10 days before irradiation.

The observation that the anemia of radiation is less severe in the animals conditioned with estrogen may also be significant in explaining the improved survival. Jacobson (8) has reported that male and female mice treated with repeated small doses of estradiol benzoate (16 μ g. weekly) sufficient to produce a partial replacement of the bone marrow by endosteal new bone formation exhibit an anemia of question-

able significance and no appreciable alteration in the other blood constituents. The absence of frank anemia under these conditions may be due to the fact that some hyperplastic marrow exists between the spicules of invading bone and that, in addition, extramedullary hemopoiesis is more marked than normal in the spleen and liver (8). Although the single large injection of estradiol employed in our experiments did not influence erythrocyte levels in the nonirradiated mice, it is possible that hemopoietic tissue is, nevertheless, rendered more resistant to irradiation as a result of the estrogen or that extramedullary hemopoiesis becomes more evident under these conditions in response to destruction of the marrow by x-rays.

SUMMARY

The observation of Treadwell and her co-workers that pretreatment with estradiol benzoate improves the survival of male mice after lethal x-irradiation has been confirmed. It has been demonstrated further that the estrogen protective effect occurs in female mice as well and that Benzestrol, but not progesterone and testosterone, is also effective. The time of injection of estrogen relative to the time of irradiation is critical, for estradiol is most effective when it is given 10 days before the exposure. Estrogen injection 5 or 15 days before irradiation also protects but administration at other intervals fails to increase survival and, indeed, may even potentiate toxicity. Estradiol in dosage sufficient to protect against x-rays does not influence survival after poisoning with a nitrogen mustard.

Estradiol (0.166 mg/mouse I.M.) increases adrenal weight and accelerates thymic involution but has no appreciable effect on weight of the spleen, inguinal nodes or kidneys. Although the increase in adrenal weight after x-irradiation is somewhat greater in mice pretreated with estrogen, the radiation-induced involution of spleen, thymus and inguinal nodes is not altered by the treatment. Experiments are cited which indicate that presence of the adrenals is not essential for the protective action of estrogens.

A leukopenia with maximal depression around 10 to 14 days after estrogen injection has been observed. The reduction in heterophils is more marked and somewhat more consistent than the lymphocyte response. Little change is noted in erythrocyte count following the single injection of estradiol. Leucocytes are decreased to the same levels in both the estrogen-treated and control irradiated animals, although maximal depression and recovery occur earlier in the former. The more rapid recovery of leucocytes in irradiated mice which receive a prior injection of estrogen resides in the heterophil component. The anemia of radiation is also less severe in the estrogen-treated animals.

Significantly, the maximal estrogen protective effect is observed when mice are irradiated during their leukopenic period. That there is some correlation between heterophil response and lethal effect is indicated in our finding that a rapid recovery of heterophils but not of lymphocytes occurs in mice treated with estradiol 10 days before x-irradiation. The possibility is considered that estrogen stimulation renders myelopoietic tissue more resistant to x-rays, perhaps because these tissues are in a proliferative phase during or shortly after the irradiation.

The authors gratefully acknowledge the technical assistance of Miss Eugenia Jackson.

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RELATION OF FOOD INTAKE TO GROWTH-DEPRESSING ACTION OF NATURAL AND ARTIFICIAL ESTROGENS¹

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BOTH natural and artificial estrogens have been demonstrated to depress growth in rats and mice (1-3). The mechanisms through which this is accomplished have not been adequately clarified. It has been suggested that estrogens may reduce anterior pituitary secretion of growth hormone (2, 3) and thyrotrophic hormone (4), or increase the secretion of adrenocorticotrophic hormone (5), any of which could result in growth depression. Non-pituitary channels may also be involved since it has been shown that estrogens can reduce body weight in hypophysectomized rats (6), inhibit important enzyme systems in the body (7) and possibly exert inhibitory effects on the nervous system (8).

The relation of estrogens to appetite has been largely overlooked, and only a few fragmentary reports have appeared on this subject. Cameron *et al.* (9) noted that rats fed estradiol appeared to eat less food, while Noble (10) observed that estrogens may reduce fluid intake in rats. The artificial estrogens, benzestrol, diethylstilbestrol and hexestrol have been reported to inhibit appetite, respectively, in normal rats (11), alloxan diabetic rats (12) and sheep (13). In goats, Meites and Turner (14) found that a dose of diethylstilbestrol which was just sufficient to induce a definite reduction in daily milk yield also elicited a definite decrease in food and water intake.

The purpose of these experiments was to determine *a*) to what extent estrogen-induced depression of growth rates in rats was due to a voluntary reduction in feed consumption and *b*) whether there were any differences in this respect between an artificial estrogen, diethylstilbestrol, and two natural estrogens, estrone and estradiol.

METHODS

One hundred female albino rats of the fast-growing Michigan State College strain and 30 female albino rats of the slower-growing Sherman strain were used in these experiments. The three hormones used, diethylstilbestrol, estrone and estradiol,² were dissolved in corn oil and injected in volumes of 0.1 ml. daily for periods of either 30 or 75 days. Diethylstilbestrol was given in dosages of 0.001 mg., 0.01 mg. or 0.1 mg. daily, and estrone and estradiol were given only at the rate of 0.1 mg. daily.

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² Diethylstilbestrol, estrone and estradiol were kindly supplied, respectively, by Dr. D. F. Green of Merck and Co., Inc.; Dr. D. W. MacCorquodale of the Abbott Laboratories; and Dr. Erwin Schwenk of the Schering Corporation.

Control rats were injected with 0.1 ml. of blank corn oil daily in order to rule out effects which might be attributable to the oil.

The food and water consumption of each group of rats was accurately measured every day, and body weights were recorded every three days. The same quantities of food and water consumed daily by the diethylstilbestrol-treated rats were given to non diethylstilbestrol-treated rats (pair-fed controls) in order to determine the extent of growth inhibition which could be accounted for by anorexia alone. All

TABLE 1. EFFECTS OF NATURAL AND SYNTHETIC ESTROGENS ON FOOD INTAKE AND BODY GROWTH OF RATS

NO. OF RATS	TREATMENT	DAYS	AV. ORIG. BODY WT.	AV. FINAL BODY WT.	AV. INCREASE IN BODY WT.	AV. DAILY FOOD INTAKE	AV. DECR. IN FOOD INTAKE
			gm.	gm.	gm.	gm.	%
<i>Michigan State College Rats—Diethylstilbestrol Series</i>							
			-I-	-I-			
10	Controls—fed <i>ad lib.</i>	30	145.0 ± 5.10	193.0 ± 6.41	48.0	10.9 ± 0.23	
10	0.001 mg. stilb. daily	30	143.5 ± 4.87	174.0 ± 5.93	30.5	9.7 ± 0.32	11.0
10	Pair-fed controls	30	143.0 ± 4.72	175.0 ± 6.30	32.0	9.7	11.0
10	0.01 mg. stilb. daily	30	143.8 ± 5.02	156.5 ± 5.01	13.7	8.8 ± 0.40	19.2
10	Pair-fed controls	30	143.5 ± 3.81	162.5 ± 4.42	19.0	8.8	19.2
10	0.1 mg. stilb. daily	30	154.0 ± 6.44	159.0 ± 6.22	5.0	7.8 ± 0.57	28.4
10	Pair-fed controls	30	149.5 ± 5.65	164.0 ± 4.06	14.5	7.8	28.4
<i>Michigan State College Rats—Natural Estrogen Series</i>							
10	Controls	75	147.0 186.0 ¹ ±4.09 ±6.01	223.0 ± 7.15	39.0 ² 76.0	10.4 ± 0.19	
10	0.1 mg. estrone daily	75	148.5 184.0 ±3.37 ±4.92	201.0 ± 5.97	35.5 52.5	10.6 ± 0.14	None
10	0.1 mg. estradiol daily	75	146.0 185.0 ±3.15 ±3.01	202.0 ± 3.89	39.0 56.0	11.0 ± 0.16	None
<i>Sherman Rats</i>							
10	Controls	30	137.0 ± 5.32	182.5 ± 4.76	45.5	10.2 ± 0.30	
10	0.1 mg. estrone daily	30	138.5 ± 4.87	167.0 ± 7.03	28.5	9.6 ± 0.29	5.8
10	0.1 mg. stilb. daily	30	135.5 ± 4.53	143.0 ± 6.35	7.5	8.0 ± 0.29	21.5

$$-I- \text{ Standard Error of the Mean} = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

¹ Average body weight at 30 days.

² Average increase in body weight at 30 days.

animals were maintained in an air conditioned room at a constant temperature of 74 degrees Fahrenheit.

RESULTS

The data are summarized in table 1. The average daily water consumption is not included since it closely paralleled food intake in all cases. It can be seen that the largest dosages of diethylstilbestrol used, 0.1 and 0.01 mg. daily, elicited the greatest inhibition of growth and appetite (figs. 1 and 2). The growth and appetite inhibiting

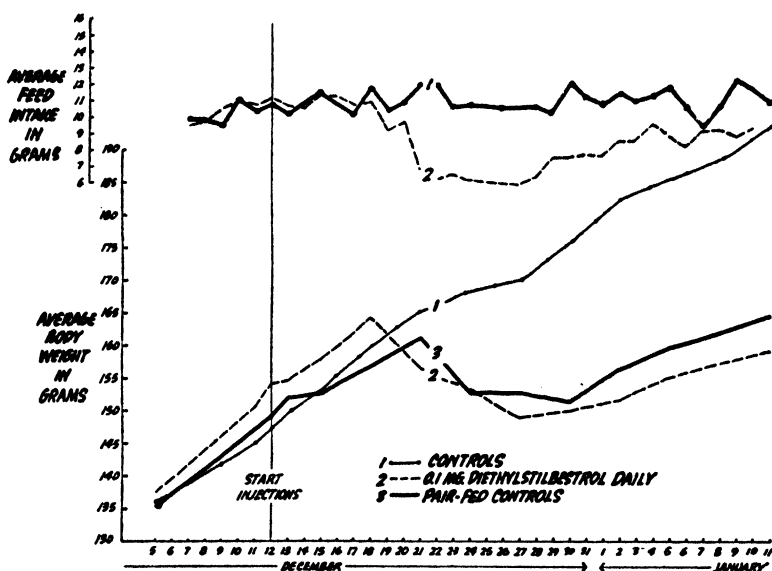
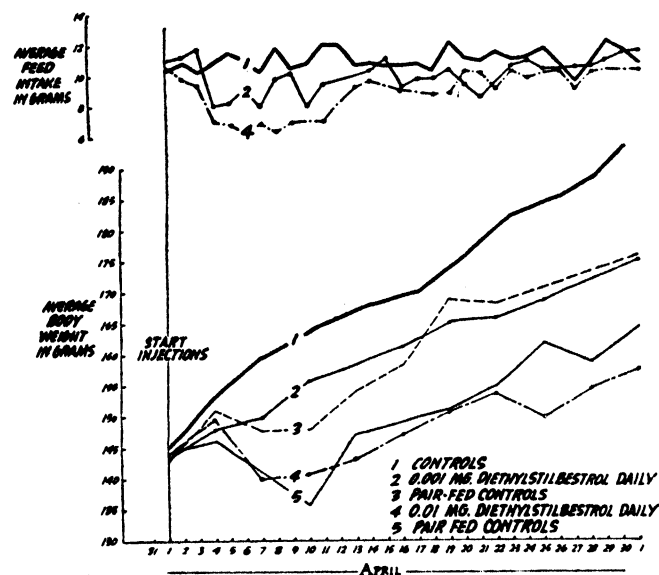


Fig. 1 (upper). EFFECTS OF INJECTIONS OF 0.001 mg. and 0.01 mg. of diethylstilbestrol daily on growth rate and food intake of Michigan State College rats. Note the direct relationship between hormone dosage, growth rate and food intake, and the parallel growth rates of the pair-fed controls.

Fig. 2 (lower). EFFECTS OF INJECTIONS OF 0.1 mg. of diethylstilbestrol daily on growth rate and food intake of Michigan State College rats. Note the parallel growth rate of the pair-fed controls.

effects of diethylstilbestrol were generally more drastic during the first two weeks than during the last two weeks of the experiment. The growth rate of the pair-fed control groups, limited in each case to the same quantities of food and water con-

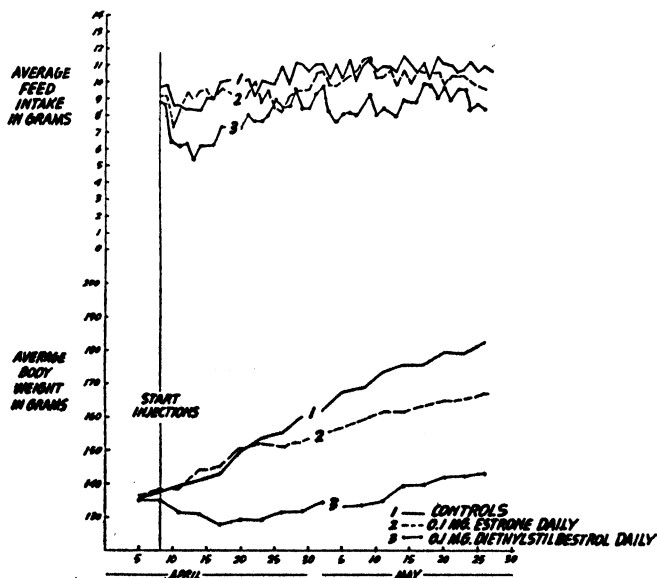
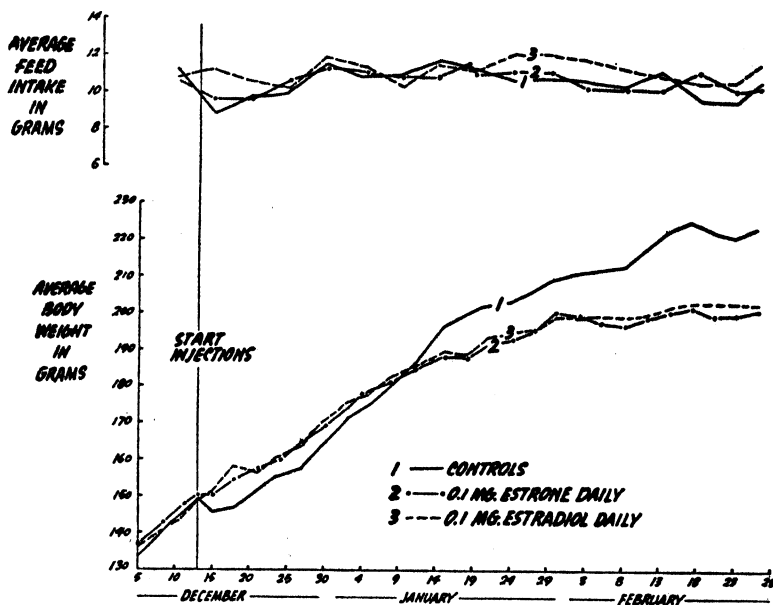


Fig. 3 (upper). EFFECTS OF INJECTIONS of 0.1 mg. of estrone and estradiol daily on growth rates and food intake of Michigan State College rats. Note that the growth rate of these rats was not reduced until after about 30 days of estrogen treatment, and that food consumption was not decreased at any time.

Fig. 4 (lower). EFFECTS OF INJECTIONS of 0.1 mg. of estrone and diethylstilbestrol daily on growth rate and food intake of Sherman rats. Note that diethylstilbestrol induced almost immediate decreases in growth rate and appetite, whereas estrone exerted a delayed and lesser growth-inhibiting effect without significantly altering food intake.

sumed by each of the diethylstilbestrol-treated groups, approximately paralleled the growth rate of the latter. This indicates that most of the growth depression exerted by the three levels of diethylstilbestrol can be accounted for on the basis of reduced food and water intake alone.

In the natural estrogen series (fig. 3) the rats which received 0.1 mg. of estrone or estradiol daily showed no reduction in growth rate until after 30 days, and no decrease in appetite throughout the entire 75 days of the experiment. It is obvious that the growth-inhibiting effects of the least amount of diethylstilbestrol used, 0.001 mg., were more marked than 100 times that quantity of either of the two natural estrogens. These data indicate that growth reduction can be induced with natural estrogens without lowering daily food intake.

The comparative effects of estrone and diethylstilbestrol on the slower growing Sherman rats are illustrated in figure 4. Here, as in the faster growing Michigan State College rats, the growth-inhibiting action of diethylstilbestrol was more drastic and occurred earlier than in the estrone-treated animals. An average daily reduction in food consumption of 21.5 per cent was induced by the diethylstilbestrol, while a small and probably insignificant decrease of 5.8 per cent occurred in the estrone-treated rats.

DISCUSSION

The results of these experiments point to the conclusion that diethylstilbestrol can curtail growth (and perhaps gonadal function and lactation) in rats principally by decreasing appetite, while natural estrogens can inhibit growth without any corresponding decrease in appetite. The possibility remains that larger doses of natural estrogens than those used in these experiments could depress food intake, or that rats of different age groups may be more susceptible to the natural estrogens. Some preliminary experiments by the author also indicate that ovariectomized rats are much more responsive than intact rats to the growth-depressing action of estrone, but there is no alteration in daily food intake. It is apparent that factors other than decreased appetite are involved in explaining reduced growth in rats given natural estrogens.

It is interesting to consider how diethylstilbestrol depresses appetite in rats. Both indirect and direct effects seem possible. In goats, Meites and Turner (14) demonstrated that the lactation-inhibiting action of diethylstilbestrol could be completely overridden by the simultaneous administration of thyroxine. This might be considered as indirect evidence that the artificial estrogen reduces thyroid hormone secretion via the pituitary, which in turn results in decreased metabolism and food intake. However, it has also been shown that a restricted food allowance itself is followed by reduced thyroid hormone secretion (15, 16), leaving unanswered the question as to whether appetite or thyroid depression comes first.

Another hypothesis has been suggested by Meites and Turner (14) to explain the reduction in food consumption following administration of artificial estrogens to lactating goats, namely that a vitamin deficiency may be created thereby. These workers noted that the decrease in milk yield following hexestrol administration could be counteracted by feeding extra vitamins in the form of fresh-cut green grass.

Ershoff *et al.* (17, 18) also observed that in immature rats fed massive doses of alpha estradiol, the inhibition of ovarian development could be counteracted by feeding yeast or desiccated whole liver. Preliminary data from our laboratory (19) indicate that the depressing effects of diethylstilbestrol on growth and appetite in rats can be partially overcome by adding vitamin B₁₂ to the diet, while thiamin and brewer's yeast appear ineffective in this respect.

SUMMARY

The relation of appetite to the growth-depressing action of diethylstilbestrol estrone and estradiol was determined in 130 female rats of the Michigan State College and Sherman strains. Diethylstilbestrol was injected in doses of 0.001, 0.01 or 0.1 mg. daily into three groups of rats, and food and water consumption were recorded daily. Three groups of control rats were fed the same amounts of feed and water consumed daily by the diethylstilbestrol-treated animals. The two natural estrogens were injected into several groups of rats in doses of 0.1 mg. daily, and food and water intake were similarly recorded.

All levels of diethylstilbestrol decreased growth and food and water intake, with the larger doses inducing the greater inhibitory effects. The growth rates of the paired control groups paralleled those of the diethylstilbestrol-treated groups, showing that the growth-inhibiting effect of this hormone was due mainly to its ability to depress appetite. The natural estrogens were less effective in reducing growth and did not decrease food consumption. The possible mechanisms through which the natural and artificial estrogens exert their different effects on growth or appetite are discussed.

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INFLUENCE OF INDUCED HYPO- AND HYPERTHYROIDISM ON VITAMIN E REQUIREMENT OF CHICKS¹

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THE effects of feeding small amounts of synthetic thyroprotein to chickens are currently being investigated in this laboratory. In one particular trial, it was observed that young cockerels fed a conventional diet supplemented with synthetic thyroprotein spontaneously developed E-deficiency symptoms while birds used as controls remained normal (1). Vitamin E deficiency symptoms are not unusual in growing chickens fed a conventional diet, especially if the fats in the diet become rancid. But in the present case this suggests that the diet supplied marginal amounts of vitamin E and that the mild hyperthyroidism induced by the feeding of synthetic thyroprotein may have increased the birds' vitamin E requirement.

A review of the literature did not yield information on the relationship of thyroidal activity to vitamin E requirement. This was not unexpected because it is rather difficult to obtain definitive vitamin E deficiency symptoms in mammals. The growing chick, however, is an excellent subject for E-deficiency studies because it is relatively simple to produce the characteristic symptoms—ataxia and cerebellar hemorrhage (2, 3). Therefore, any effect of hyperthyroidism on vitamin E requirement would be more readily observed in chicks than in other experimental animals.

The present communication describes the effects of experimentally induced hyper- and hypothyroidism in chicks fed purified diets containing known amounts of vitamin E.

EXPERIMENTAL

Two hundred four-day-old White Leghorn males were divided at random into 12 groups of 17 chicks, and each group was confined in a separate compartment of an electric battery brooder provided with wire mesh floors. Two groups served as replicates for each of the six diets fed.

Dam's (3) basal diet no. 190³ was modified to provide the following diets:

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³ U.S.P. XII Salt Mixture plus 0.0012 gm. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per 100 gm. of basal diet was used instead of the mixture employed by Dam.

vitamin E: soybean (or corn) oil and 'crude' casein. Furthermore, the time interval (one week) was of too short duration to sufficiently deplete the endogenous vitamin. Dam (3) was unable to produce deficiency symptoms in less than 12 days, and in the present study the first chicks did not show symptoms until the end of the second week on a deficient diet.

The excessive incidence of perosis which occurred in hyperthyroid chicks fed the E-sufficient diet suggests that elevated metabolism may also increase the requirement for other substances. Manganese (6), choline (7) or biotin (8, 9) deficiency is known to cause perosis.

One of the most interesting implications of this study lies in its possible use in the biological assay of vitamin E. The use of hyperthyroidism to accelerate depletion of endogenous vitamin E would make possible the employment of 'basal' animals for assay purposes in the manner used by Robblee *et al.* (10) in studying an unidentified chick growth factor. These workers found that synthetic thyroprotein increased the requirement for the growth factor and hence could be used to produce depleted animals for test purposes.

SUMMARY

Experimental hyperthyroidism increases, whereas induced hypothyroidism decreases the rate of utilization (depletion) of endogenous vitamin E in chicks fed an E-deficient diet. There is some evidence that hyperthyroidism may also increase the requirement for choline, biotin or manganese. The findings of the present work imply that supplementary vitamin E may be indicated in natural or induced hyperthyroidism, or in cases of thyroid therapy. The technique used in this study would appear to be valuable in the biological assay of vitamin E. Hyperthyroidism, used to accelerate depletion of endogenous vitamin E, would make possible the employment of 'basal' animals for assay purposes.

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EFFECT OF SEVERE STRESS UPON THYROID FUNCTION

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MANY factors are known to increase the metabolic rate; among these are exercise, extremes of temperature, insomnia, food, fever and a great variety of emotional disturbances. The mechanisms by which the increases are effected have been incompletely elucidated. One of the probable contributory factors which was investigated early was the rôle of adrenalin. Krantz and Means (1) found that a single subcutaneous injection of 0.625 mg. of adrenalin raised the metabolism of normal people to a level of about plus 25 per cent. Marine and Baumann (2) observed in the rabbit and cat with an intact thyroid that a transient symptom-complex was produced by injury to the adrenals, which closely resembled Graves' disease. More recently, Soffer and associates (3) found that prolonged administration of adrenalin to dogs was followed by hyperplastic changes in the thyroid. Bioassay of the serum of thyroidectomized dogs after adrenalin administration showed an increase in circulating thyrotropin. Although adrenalin probably exerts a calorogenic effect, independent of changes induced in the thyroid, the latter are doubtless of significance under certain circumstances.

The influence of the cervical sympathetic nervous trunk upon the thyroid was investigated by Cannon, Binger and Fitz (4) by anastomosing the distal end of the trunk with the phrenic nerve. There resulted an increased oxygen consumption, nervousness, weight loss and cytological changes of hyperthyroidism. Friedgood and Cannon (5) upon investigating the phenomenon concluded that the thyroid stimulation was mediated through the pituitary. Uotila (6) found that extirpation of various parts of the cervical sympathetic system failed to produce much direct effect on the cytology or function of the thyroid.

Uotila (7) as well as Wolf and Greep (8) reported that exposure of rats to cold increased the activity of the thyroid, provided that the pituitary was present. Uotila showed that it was necessary also to have the hypophyseal stalk intact.

Starr and Roskelley (9) observed that in rats exposed to temperatures of 12° to 17° C., the thyroid cells initially became hypertrophied but later decreased markedly in size when the exposure was extended to 56 days. Dempsey and Astwood (10) concluded on the basis of indirect evidence that the release of thyroxin from the thyroid increased when the animal was exposed to cold and decreased when he was in a heated environment.

Leblond and collaborators (11), using radioiodine to study thyroid function, concluded that in rats exposed to cold (0° to 2° C.) for various intervals there was questionable stimulation of the thyroid within the first 3 days, definite after 7 days, maximal at 26 days, and none at 40 days. At the time of maximal stimulation by the cold the fixation of radioiodine was 2.7 times that of the control.

Exposure to heat was found to lessen thyroid activity. The decrease was observed as early as after one day, and it persisted for at least 26 days.

We have investigated in rats, the effect of starvation, heat, cold, adrenalin, adrenal cortical extract, typhoid vaccine, and trauma upon the distribution of radioiodine in the thyroid gland and in the serum. The content of radioiodine in urine and the total and protein-bound radioiodine of serum were determined in a series of patients while they were undergoing pronounced stress, and constitute a separate report.

METHODS

The rats were of the Sprague-Dawley strain, and almost all of them were male. They were raised in the Thorndike Memorial Laboratory and were maintained on a diet of Purina Laboratory Chow, supplemented weekly with carrots and lettuce. At the time of the various experiments the rats were from 60 to 70 days of age. From 4 to 6 animals were used to test any one phase of each experiment.

The tracer dose of radioiodine (I_{131}) consisted of 100 microcuries, except where otherwise stated. In all instances it was given subcutaneously, usually in 1 cc. of saline. Caution was taken to pinch the skin at the site of injection very firmly in order to prevent leakage. The rats were killed by a blow on the head, except in the ones from which blood was taken; these were killed by a guillotine method. In one experiment sodium pentothal anesthesia was used but varying degrees of shock caused such marked discrepancy in the results that they were unreliable. The thyroid glands were removed quickly, weighed upon a torsion spring balance, digested in 20 per cent potassium hydroxide and analyzed for the radioiodine content. The determination of radioiodine in serum consisted of placing 0.5 or 1 cc. of serum in a bottle cap with one drop of dupanol. After drying in an oven the cap was placed under a Geiger-Müller tube and counts were made. In determining the PBI¹ 1 cc. of serum was pipetted into a 15 cc. centrifuge tube. Then 9 cc. of 10 per cent trichloroacetic acid was added while the solution was stirred vigorously. After centrifugation the supernatant fluid was decanted. The precipitate was washed three times with 5 cc. portions of 10 per cent trichloroacetic acid, transferred quantitatively to bottle caps, dried at 100°C. and then isotope counts were made.

Animals exposed to cold were kept at 5° C., while those exposed to heat were kept at 38° C.; the other rats were kept at 26° C. Other conditions of the experiments are described as each is presented.

Experiments I and II. Each animal was given a tracer dose of radioiodine one hour before it was killed. It can be observed in figure 1 that in animals that had been fasted, exposed to cold or heat, or given adrenalin², the concentration of radioiodine in the thyroid gland was less than normal. The changes were greater in animals subjected to heat or starvation for two days than in those so treated for three days. The rats given adrenalin one hour before radioiodine was injected, and again at the time the radioiodine was given, had a lower concentration of isotope in the thyroid than did the ones given adrenalin only with the radioiodine.

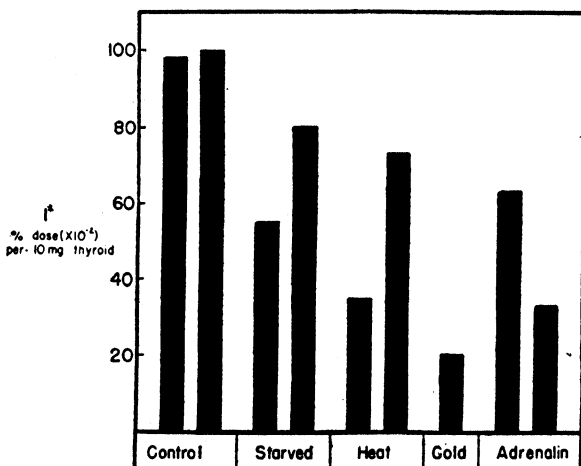
¹ Protein bound iodine.

² The solution of aqueous adrenalin contained 1 mg/cc. The oily preparation contained 2 mg/cc.

Experiment III. In this experiment each animal was killed 2 hours after radioiodine was injected. The rats were fasted for 3 days, or exposed to heat or cold for 2 days. One group of normal rats was given radioiodine followed one hour later by 0.2 cc. of adrenalin in oil, and 0.1 cc. of aqueous adrenalin. As shown in figure 2, there was a subnormal concentration of radioiodine in the thyroid following adrenalin therapy, starvation and exposure to heat or cold.

Experiment IV. In this experiment, the radioiodine was given 24 hours preceding the death of the rat. No food was given during the last 4 hours of the experiment. One group was given 0.2 cc. of adrenalin 2 hours before death. Another group received the same dosage 48, 24 and 2 hours preceding death. Five rats were subjected to trauma by applying a rubber band on the left hind leg for 15 hours 2 days before death, on the right hind leg for 2 hours on the next day, and on the right front leg for one hour beginning 2 hours before death. At least one leg of each animal became

Fig. 1. RESULTS OF 2 EXPERIMENTS are presented. The 2nd, 4th and 6th columns are results of the 2nd experiment. The only difference in the characteristics of the 2 experiments is that the 2nd one was conducted for 3 days while the first one was for 2 days. One group of rats, shown in the last column, was given 0.2 cc. of adrenalin one hour before radioiodine and again with it; the other group was given 0.2 cc. of adrenalin concomitantly with the isotope. Each column represents average result obtained with from 3 to 6 rats.



gangrenous. Four rats were each given 0.5 million killed typhoid bacilli intraperitoneally 2 days before decapitation; the same sized dose was given the following day, and one million organisms were administered 2 hours before the rats were killed.

Bilateral adrenalectomy was performed on 15 rats 2 days before death; they were given saline as drinking water. Five received no other treatment; 5 were given 0.5 cc. of lipoadrenal extract (Upjohn) at 48, 24, and 3 hours, respectively, before death; 5 were given 0.2 cc. of adrenalin in oil at 48, 24, and 2 hours, respectively, before death.

As seen in figure 3, the normal animals which received adrenalin, trauma or typhoid vaccine were found to have distinctly less protein-bound radioiodine in the serum than did the untreated ones. On the other hand, the concentration of isotope in the thyroid was hypernormal. The studies with the adrenalectomized animals demonstrated less protein-bound radioiodine in the ones treated with adrenal cortical extract or adrenalin than in the controls, but there were no statistically significant differences in the quantity of isotope in the thyroid. All of the adrenalectomized rats had more radioiodine in the thyroid than did normal animals.

Comments. The results that have been presented thus far show that following the various types of stress the concentration of radioiodine in the thyroid of normal rats became subnormal when the isotope was given one or two hours before thyroidectomy, but hypernormal when it was administered 24 hours before. The serum protein-bound radioiodine was found to be subnormal in the animals subjected to severe stress and given radioiodine 24 hours preceding death. Since there is cytological evidence (7) of increased thyroid activity within 24 hours of exposure to stress, an increased rate of uptake of radioiodine by the thyroid would be expected.

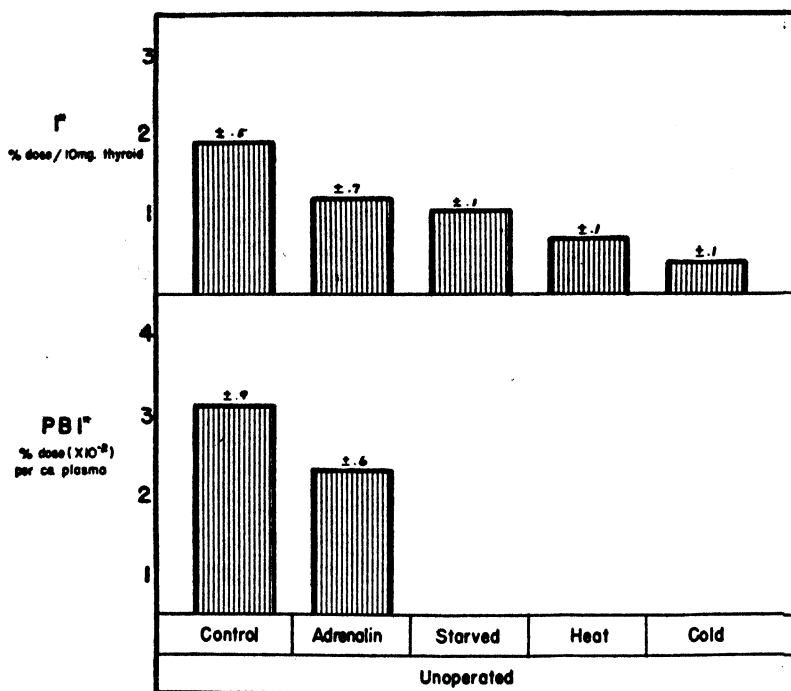


Fig. 2. EACH COLUMN presents the mean value obtained with from 3 to 6 rats; standard deviation from the mean is indicated.

However, with the increased production of thyrotropin there would occur an increased rate of manufacture of thyroid hormone and of its transfer into the blood stream. The increased metabolic rate associated with the severe stress could be expected to increase the rate of breakdown of thyroid hormone. Since the quantity of radioiodine in the thyroid and in protein-bound form in the serum is a balance of the factors discussed above as well as the quantity of radioiodine excreted or existing in sites other than the thyroid or serum, with the information available, it is difficult to evaluate adequately the individual factors involved. In order to help elucidate the problem the following experiment was conducted.

Experiment V. Each of 39 rats was injected with 8 microcuries of radioiodine every day for 3 days. They were given 16 microcuries upon the fourth day and killed

on the fifth. All of these animals were fasted for from 15 to 18 hours before death. The thyroid gland of 8 animals was removed, using ether anesthesia; each of these rats was killed 3 hours later. Four of them were given 0.3 cc. of adrenalin in oil immediately after thyroidectomy and 0.2 cc. of aqueous solution of adrenalin 20 minutes before death. The purpose of this phase of the experiment was to determine the effect of adrenalin, and the hypermetabolism which it produces, upon the utilization of the protein-bound radioiodine of the serum. The effect of the operative

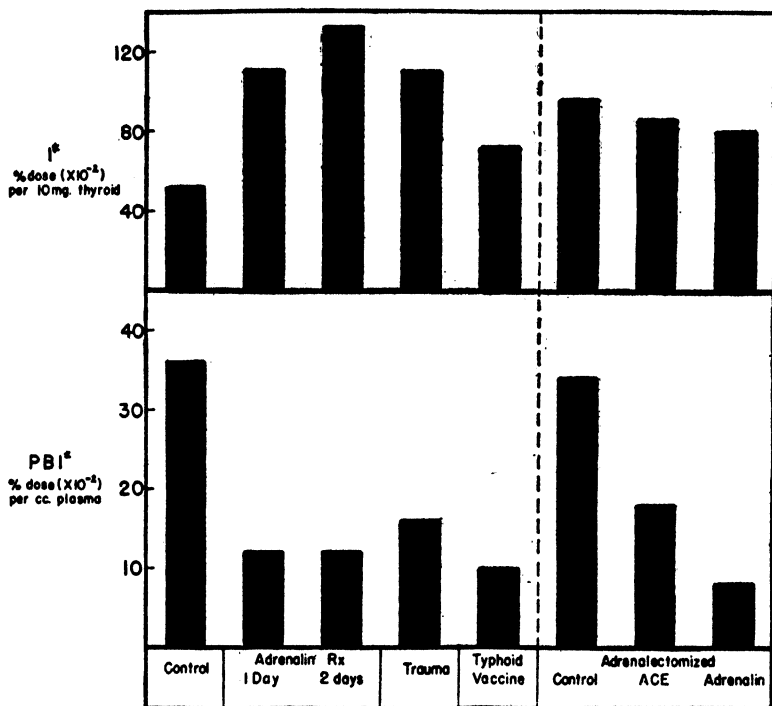


Fig. 3. EACH COLUMN presents mean value obtained with from 4 to 6 rats; standard deviation from the mean is indicated.

procedure upon the thyroid was evaluated by a sham operation. The muscles anterior to the thyroid were cut and the gland was completely exposed.

In order to permit the thyroid to evacuate its hormone during stress but to antagonize additional formation of hormone, 100 mg. of sodium thiouracil was given in 2 cc. of saline, intragastrically, to 8 rats, 5 hours before they were killed. Three hours before death, each of 4 of these animals was given 0.3 cc. of adrenalin in oil; 0.2 cc. of an aqueous solution of adrenalin was given 20 minutes before decapitation. The remainder of the 39 rats served as controls. Determinations were made in each of the 39 rats of the total radioiodine concentration in the thyroid and in the serum, as well as in the protein-bound fraction of the latter.

None of the treatments caused an unequivocal change in the radioiodine content of the thyroid. The total and the protein-bound iodine of the plasma was less in the

two groups of rats given adrenalin than in any of the others. In the group treated with thiouracil it is presumable that synthesis of the hormone had ceased, but it was still possible for adrenalin, by means of increasing thyrotropin production (3), to increase the rate of transfer of thyroid hormone into the blood and to increase the rate of metabolism of protein-bound radioiodine. In the rats that were thyroidectomized, only the latter effect would be anticipated. However, it is quite possible that, with the manipulation of the thyroid gland, more protein-bound radioiodine was liberated from the thyroid than was induced by the increase in thyrotropin. The thyroidectomized control group was not found to have an increase in thyrotropin nor

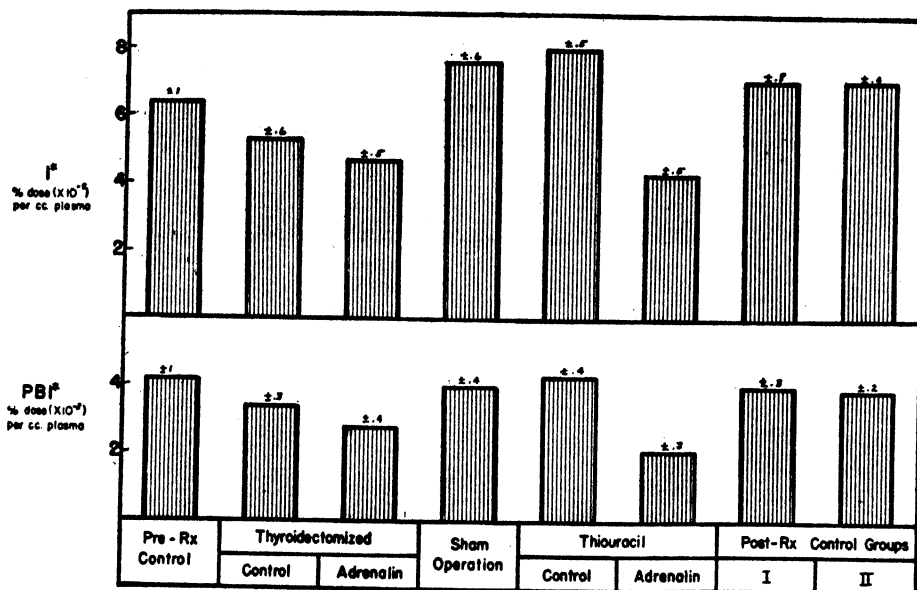


Fig. 4. THE GROUP designated as 'pre-treatment control' were killed just before thiouracil or adrenalin was administered, or thyroidectomy was performed. The rats in the post-treatment control groups were killed simultaneously with the ones that had been treated. Each column presents the mean values obtained with 4 or more rats; standard deviation from the mean is indicated.

in the protein-bound radioiodine. Although it is probable that these animals were given extra adrenalin endogenously, the increased rate of utilization of the thyroid hormone may have helped to balance the increased transfer of 'thyroxin' from the thyroid to the plasma. Just what effect the different forms of treatment had on the excretion of radioiodine or its distribution in other parts of the body was not determined.

DISCUSSION

It has been shown that various types of stress affect the quantity of radioiodine in the thyroid and in the serum, particularly the protein-bound fraction. However, it is difficult to determine the extent to which various phenomena are involved in producing the alterations in iodine metabolism. The concentration of isotope in the thyroid is, of course, a balance between the quantity of it taken from the blood by the gland and the amount released.

The rate of these changes depends upon the gradient of iodine between the blood and thyroid, thyrotropic hormone activity, rate of utilization of the thyroid hormone and other factors. Adrenalin increases the rate of release of thyrotropin from the pituitary, as apparently does the exposure to cold. Whether other alarming stimuli increase thyrotropin release has not been proved, although it seems logical that many of them would increase it. Apparently various stresses lead to an increased production of thyroid hormone, but there was also an increased rate of utilization of it. Neither the presence of the adrenals nor of the thyroid was necessary for adrenalin to decrease the protein-bound radioiodine of the plasma.

The results of Leblond and colleagues (11) show that the duration of exposure to cold and the dosage of iodine are important in determining the concentration of isotope in the thyroid. When in their experiments 0.2 microgram of iodine was used, a decrease in the radioiodine in the thyroid was found during the first few days of exposure. When 5 micrograms were used, there was an apparent increase after a seven-day exposure and maximal increase after a 26-day exposure, but no increase after a 40-day exposure.

SUMMARY

In rats, the concentration of radioiodine by the thyroid and its distribution in the serum were found to be influenced by adrenalin, trauma and typhoid vaccine. Adrenalin or adrenal cortical extract decreased the quantity of protein-bound iodine in the serum of adrenalectomized animals. Adrenalin decreased it in normal and thyroidectomized rats. Fasting, or exposure to cold (5° C.) or to heat (38° C.) for intervals of three days was associated with a subnormal concentration of radioiodine in the thyroid.

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METABOLISM OF DOGS DURING INTOXICATION FROM AGENIZED WHITE WHEAT FLOUR¹

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IT HAS been repeatedly demonstrated that various protein materials which have been treated with nitrogen trichloride ('Agene') when fed to animals will cause signs of intoxication, principally epileptiform convulsions (1, 2). These signs have been adequately described in previous communications (3, 4). The present investigation sought to determine some of the metabolic changes accompanying such intoxication. Feeding experiments upon a variety of animals have demonstrated a marked species specificity for this type of poisoning, and of all animals tested, the dog is outstanding in its susceptibility to the toxic agent. For this reason this animal was employed throughout the present study.

METHODS

Six healthy, adult, mongrel dogs of either sex, and with body weights from 5 to 9 kg. each, were studied. They were housed in individual metabolic cages kept in an air-conditioned animal house at 73°F. Use of these cages permitted separate quantitative measurement of dietary and fluid intake and of the excretion of urine and feces. The animals were allowed freedom of activity within the cages but not outside.

Diets previously shown to be nutritionally adequate for dogs (3) were fed *ad libitum*. The animals consumed a daily average of 35 grams of food per kilogram of body weight and maintained their body weights well throughout the course of the study. Seventy-five per cent of the diet consisted of white wheat flour, which was untreated during the preliminary control period. During the period of intoxication, the untreated flour was replaced by flour treated with nitrogen trichloride ('Agene') to the extent of 10 grams of NCl_3 per 100 pounds of flour. The diet was otherwise unchanged.

Metabolic observations were made on two separate days of a preliminary control period which lasted 10 days. During the phase of intoxication, observations were made from day to day after clinical evidence of poisoning became unequivocal. Death usually ensued within 15 days after the introduction of feeding with agenized flour, but no animal was studied while moribund.

Daily records were kept of food and water intake, urine volume and specific gravity, and body weight. On the appropriate days, blood samples were taken from the external jugular vein, with the dog under light intravenous pentobarbital anesthesia. When the animals were judged to be approaching a terminal state, electroencephalographic records were made in a manner previously described (3). This procedure served to confirm the clinical diagnosis of intoxication.

The following qualitative tests were made on the urine of all animals on alternate days throughout the study: reducing substances, pH, protein, bile pigments, ketone bodies, blood (benzidine) and microscopic formed elements. The urine was analyzed quantitatively for the following substances: total nitrogen, urea nitrogen, creatinine, calcium and phosphorus. Formed elements of the blood

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¹ The opinions expressed in this paper are those of the authors, and do not necessarily represent the official views of any governmental agency.

were counted and hemoglobin and hematocrit were measured. Samples of whole blood or serum were analyzed quantitatively for glucose, non-protein nitrogen, albumin, globulin, urea nitrogen, phosphorus, calcium, acetylcholine and cholinesterase. Circulating serum acetylcholine was determined by the method of Wait (5), a procedure which uses the isolated heart of the mollusc *Venus mercenaria*. The serum cholinesterase was determined by the method of Michel (6) in aliquots of the same samples of sera as used for estimating acetylcholine. Cholinesterase activity is reported in units of decrease of μH per hour in a veronal buffer containing acetylcholine bromide as the substrate.

Liver function was evaluated in both groups of animals by three different tests that are widely used clinically; viz., cephalin flocculation, thymol turbidity and bromsulphalein retention.

RESULTS

Although there was some variation, the average animal commenced to show signs of intoxication which became pronounced by the 7th day. A terminal condition was reached in about 15 days.

TABLE 1. CLINICAL COURSE OF 6 DOGS BEFORE AND DURING INTOXICATION FROM AGENIZED WHITE WHEAT FLOUR COMPARED WITH AVERAGE SERUM ACETYLCHOLINE AND SERUM CHOLINESTERASE

PERIOD	TYPICAL CLINICAL COURSE	VALUES FOR SERUM		
		Acetylcholine	Cholinesterase units	Ratio: acetylcholine/cholinesterase
		$\mu\text{g}/100 \text{ ml.}$	$\Delta \text{ pH/hr}$	
Control ¹	Normal behavior	0.87	0.37	2
Intoxication ²				
Day 4	Intermittent periods reminiscent of 'petit mal.' Few if any convulsions	0.54	0.60	1
Day 7	Typical epileptiform, convulsions about every 4-8 hrs.	0.48	0.59	1
Day 11	Typical epileptiform convulsions about 1/hr.	1.86	0.34	5
Day 15	'Status epilepticus,' convulsions very frequent (no animal studied when moribund)	2.76	0.06	46

¹ Mean of two observations on different days.

² Observations on single days during progressive intoxication.

Alterations in the serum cholinesterase activity and in the concentration of acetylcholine in the serum were the only significant metabolic changes found in this study (table 1). The average value for acetylcholine in the control period was 0.87 $\mu\text{g}/100 \text{ ml.}$ of serum. The concentration of this substance increased progressively, following the appearance of the signs of intoxication, to attain an average final value of 3.60 $\mu\text{g}/100 \text{ ml.}$ of serum. At the same time serum cholinesterase activity decreased from an average control value of 0.35 units to a final value of 0.06 units. This decrease did not become notable until the clinical signs were well advanced.

The remaining metabolic measurements showed no consistent or biologically significant changes during the period of intoxication. Studies of the formed elements of the blood, as well as of the hemoglobin and the hematocrit, revealed no notable

variations in value between the two periods (table 2A). These values were within the normal limits for healthy dogs. Similarly, there were no striking variations in blood glucose, serum non-protein nitrogen, serum total protein, serum albumin, serum globulin, serum A/G ratio, serum urea nitrogen, serum inorganic phosphorus or serum calcium (table 2B). All of the values for these components of the blood and

TABLE 2. AVERAGE VALUES FOR HEMATOLOGY, BLOOD CHEMISTRY, AND URINARY EXCRETION PRODUCTS OF 6 DOGS BEFORE AND DURING INTOXICATION FROM AGENIZED WHITE WHEAT FLOUR

COMPONENT ¹	CONTROL PERIOD ²	PERIOD OF INTOXICATION ³
<i>A. Blood—Hematology</i>		
Erythrocytes, millions/mm. ³	4.3	4.3
Leukocytes, thousands/mm. ³	7.4	8.1
Hemoglobin, gm/100 ml.	13.2	13.5
Hematocrit, %	40.3	39.9
<i>B. Serum and whole blood chemistry</i>		
Whole blood glucose, mg/100 ml.	100	81
Serum non-protein nitrogen, mg/100 ml.	35	34
Serum total protein, gm/100 ml.	6.0	6.2
Serum albumin, gm/100 ml.	3.3	3.5
Serum globulin, gm/100 ml.	2.8	2.7
Serum A/G ratio	1.2	1.3
Serum urea nitrogen, mg/100 ml.	11.9	11.7
Serum inorganic phosphorus, mg/100 ml.	5.2	4.7
Serum calcium, mg/100 ml.	13.3	12.9
<i>C. Urine</i>		
Daily volume, ml/day	128	135
Specific gravity, water = 1.000	1.037	1.037
Total nitrogen, gm/day	3.69	3.27
Urea nitrogen, gm/day	1.70	1.65
Creatinine, mg/day	215	187
Calcium, mg/day	14.4	11.9
Phosphorus, mg/day	329	314

¹ None of the measurements listed in table 2 showed consistent or biologically significant differences between the control and intoxication periods.

² Average of 2 measurements on separate days of the control period.

³ Average of 2-4 measurements toward the end of the period of intoxication.

serum were well within the normal limits for healthy dogs. Between the control period and the test period there were no significant changes in the average daily urinary excretion of total nitrogen, urea nitrogen, calcium, phosphorus or creatinine (table 2C).

Kidney function remained normal throughout the period of intoxication. The daily volume of urine and the average specific gravity of the urine did not vary significantly between control and test periods (table 2C), and a well concentrated, slightly alkaline urine was produced at all times. No variations from the normal were

seen at any time in urinary reducing substances, acidity, protein, bile pigments, ketone bodies, free hemoglobin or microscopic formed elements.

Liver function was not disturbed at any time, as judged by normal retention of bromsulphalein in all animals, and by normal results for thymol turbidity. The cephalin flocculation was positive in all specimens of serum during both the control and experimental periods. This 'false positive' result was presumably caused by a serum protein peculiar to the dog, and not found in the serum of healthy human beings.

DISCUSSION

Previous reports from this laboratory by Silver *et al.* (3), claiming failure of dogs to concentrate urine normally when suffering from the toxic effects of agenzized protein, seem to be refuted by this study. It is believed by the present authors that this misconception arose from the fact that in the former studies the animals splashed water from their drinking pans into the urine-collecting bottles beneath the cages during convulsive seizures. Such spillage would dilute the urine, causing an apparent low specific gravity. In the present study such an experimental error was impossible and no changes in kidney function could be detected.

Hematologic and biochemical studies similar to those reported here have been conducted by Newell *et al.* (7), whose results parallel those of the present report in all instances in which similar determinations were done. In addition to the extensive negative findings reported, these workers found that during periods of intoxication there was a definite increase in both magnesium and potassium in the serum. Administration of these ions during periods of acute poisoning, however, failed to produce any intensification of symptoms. No explanation of the retention of these elements was offered by Newell *et al.* (7).

The alterations which occurred in the circulating acetylcholine and cholinesterase are the only biochemical evidence of toxic effects to be revealed by the present study. Although the changes were definite and statistically significant, the question of the specificity of such damages in this type of intoxication cannot be presently answered. Changes in the level of circulating cholinesterase are known to occur in relation to several disease processes or stress. For instance, cholinesterase is usually lowered in chronic liver disease, in hypogonadism and in the 'alarm reaction' (8-10). In the present study there was no demonstrable liver damage to explain the results. It may well be that the changes found were secondary to the convulsions, since only after the clinical signs were well advanced did the increase in acetylcholine and decrease in cholinesterase become pronounced. Nevertheless, it is of interest, in the light of their well known rôles in neuromuscular transmission, that acetylcholine and cholinesterase should undergo such changes in the presence of an unknown neurotoxin, whose chief effect in dogs is the production of convulsions.

At this writing the only biochemical changes which have been described in agene intoxication involve the blood magnesium and potassium and the acetylcholine-cholinesterase relationship described in the present communication. It follows that the toxic signs may be based upon changes of a subtle nature, possibly within a cerebral enzyme system.

SUMMARY

The metabolism of 6 dogs was studied intensively to determine what, if any, changes occur in conjunction with intoxication from the ingestion of agenzized white wheat flour. There was a large increase in serum acetylcholine and a striking decrease in serum cholinesterase activity during intoxication. These changes were progressive, being greatest toward the end of the period of intoxication. No impairment of kidney or liver function could be detected at any time, as judged by widely used clinical and laboratory criteria.

No consistent or biologically significant changes during the course of intoxication were detected in erythrocyte count, leukocyte count, blood hemoglobin, hematocrit, blood glucose, serum non-protein nitrogen, serum total protein, serum albumin, serum globulin, serum A/G ratio, serum urea nitrogen, serum inorganic phosphorus, serum calcium, urinary total nitrogen, urinary urea nitrogen, urinary creatinine, urinary calcium or urinary phosphorus.

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ENZYME STUDIES ON HUMAN BLOOD. V. ESTIMATION OF PROTHROMBIN BY AN HOMOLOGOUS-ISOLATION METHOD¹

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THIS laboratory has directed its investigations in the coagulation problem towards the thrombin-fibrinogen reaction. Thus, the effects of fibrinogen concentration and purity (1), of plasma proteins (2), and of heparin (3) on this reaction have been reported. In order that similar and other studies could be extended to the activation phase, it became apparent that a new technic for the determination of prothrombin must be developed since the one-stage (4, 5) and the two-stage (6, 7) technics did not fulfill certain requirements which included the following: homologous reagents; removal of fibrinogen without added thrombin; absence of acacia and albumin in both the activation and final reaction mixtures; a stable prothrombin fraction; and a system which could be applied in investigations of known and unknown coagulation dynamics and factors, other than prothrombin.

Reported in this communication is an homologous-isolation method for the determination of prothrombin. Fibrinogen is first removed as Fraction I by the application of the low temperature-alcohol principle of Cohn *et al.* (8) to a small volume of plasma. The supernatant fluid is then treated by a procedure similar to Mellanby's method (9) for the preparation of prothrombin concentrates except that native fibrinogen has been previously separated out. The prothrombin fraction is activated with calcium and placental thromboplastin with and without the presence of dilute fresh pooled normal plasma. The resulting thrombin is titrated with fibrinogen. Both the thromboplastin and fibrinogen reagents are of human origin and are quantitatively standardized. The final reference of potency is a thrombin preparation of the National Institutes of Health.

One unit of thrombin is defined here as that in 0.2 cc. volume which will clot 0.8 cc. of fibrinogen solution containing 0.15 to 0.3 per cent clottable protein in 15 seconds at $T/2$ 0.129, pH 7.2. One unit prothrombin is that which when fully activated at pH 7.2 25 to 28° C., with optimum concentration of calcium and known excess thromboplastin, will result in one unit of thrombin within 30 minutes. One unit of thromboplastin is the minimum required to convert one prothrombin unit to one thrombin unit.

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¹ Fibrinogen fractions were prepared in this laboratory from dried plasma processed from volunteer donors enrolled by the American Red Cross. Standard human thrombin powder was obtained through the courtesy of the Division of Biologic Control of the National Institutes of Health, Bethesda 14, Md.

The results by this new technic on 24 normal plasma samples are compared with those obtained by the thrombin method, a modification of the two-stage technic (7): Plasma is first defibrinated with thrombin; however, acacia has been omitted and homologous reagents are employed. Data on duplicate experiments with 7 other samples of plasma to evaluate the error of a single determination and of prothrombin recovery are also presented. In addition, methods for the preparation and standardization of human placental thromboplastin are described.

METHODS AND MATERIALS

Citrate-Phosphate Buffer. This buffer, $\Gamma/2$, 0.129 and pH 7.2, consists of 250 cc. 1/15 M Sorensen buffer, pH 7.1, and 75 cc. of 0.1 M sodium citrate in one liter solution (1) and unless otherwise indicated it was employed throughout this work. A glass electrode electrometer, standardized daily, was employed for all pH determinations.

Buffered 53 Per Cent Ethanol. Sufficient acetate buffer, 0.8 M, pH 4.0, was added to 53.3 per cent ethanol (8) so that when 0.72 cc. of this buffered alcohol was mixed with 4 cc. of plasma at $-3^{\circ}C.$, the final pH would be 7.2 ± 0.2 . In the present work on fresh liquid plasma, 0.8 cc. acetate buffer added to 72 cc. 53.3 per cent ethanol was adequate as evidenced by pH control experiments on separate aliquots of individual plasma samples. Similar studies should be made by each laboratory and for various types of plasma. This reagent was stored in a $-25^{\circ}C.$ refrigerator.

Preparation of Human Placental Thromboplastin. Within two hours after parturition the placenta was stripped of the cord, large blood vessels and clots; rinsed with cold distilled water; sponged with gauze; and then weighed. When not in use, the glass bowl of the Waring Blendor was placed in a $-25^{\circ}C.$ refrigerator. The temperature of the citrate-phosphate buffer and the centrifuge (International PR1) was between 0° and $+1^{\circ}C.$ throughout the following procedure. The organ was cut into small slices, blended with an equivalent weight of citrate-phosphate buffer for 3 to 5 minutes, and then centrifuged at 700 g for 15 minutes. The sediment was again extracted with one-half volume of buffer. The two extracts were combined and the volume adjusted with buffer so that 150 cc. was equivalent to 100 gm. of tissue. This *crude tissue extract*, which was found to be stable indefinitely at $-25^{\circ}C.$, was then processed according to Chargaff's principle (10). A convenient volume, usually 150 cc., was centrifuged in an angle head at 32,000 g for 30 minutes. The supernatant fluid was decanted off and the sediment was blended approximately one-half minute with buffer, volume one-half that of the original crude tissue extract, and then centrifuged at 32,000 g for 30 minutes. This step was repeated 4 to 6 times or until the supernatant fluid contained less than 5 mg. per cent protein by the sulfosalicylic acid turbidity method (11). The final sediment was lyophilized, initial temperature was $-25^{\circ}C.$ and pressure was 60 micra mercury. The resulting *crude thromboplastin powder* was weighed and stored over silica gel in a vacuum desiccator in which it was found to be stable indefinitely. *Stock thromboplastin solution* was prepared on the day of an experiment as follows: A 1 per cent suspension of the crude powder in citrate-phosphate buffer was stirred and heated at $56^{\circ}C.$ for 5 minutes and then allowed to sediment for at least 15 minutes. The fluid was pipetted off and filtered through four layers of gauze.

Standardization of the Thromboplastin Solution. To 1 cc. of diluted pooled normal prothrombin fraction, 10 units/cc., was added 0.5 cc. of varying dilutions, 1 per cent to 100 per cent, of stock thromboplastin solution and 0.5 cc. 0.025 M CaCl_2 and tested as in the isolation technic described below. The lowest concentration of the stock thromboplastin solution resulting in the maximum conversion of prothrombin to thrombin was considered the end point. Both the stock and working solutions did not clot upon addition of thrombin, did not clot fibrinogen solutions in the presence of optimum calcium concentration, and were stable for at least one working day at room temperature.

Standardization of Calcium. The optimum calcium concentration was determined by essentially the same procedure as employed for the thromboplastin standardization. To activation mixtures were added 0.5 cc. volumes of calcium chloride solutions, 0.005 to 0.100 M. That concentration, 0.025 M, resulting in maximum thrombin formation was selected as the standard reagent.

Standard Fibrinogen Solution. Fraction I, of at least 60 per cent purity in respect to fibrinogen, was prepared from human plasma by *method 6* of Cohn *et al.* (8). A 1 per cent suspension of the powder was stirred in citrate-phosphate buffer at room temperature for 20 minutes and then centrifuged for 20 minutes at 1500 g. The supernatant fluid, on which total and clottable protein was analyzed for each lot of Fraction I powder, was then diluted with buffer to 0.15 to 0.30 per cent fibrinogen, the optimum concentration as previously established (1). This standard solution did not clot at room temperature in one working day nor did the clotting time with standard thrombin vary significantly during this period.

Standard Thrombin Solution. Human thrombin from the National Institutes of Health (N.I.H.) 12.5 U/mg., was dissolved in citrate-phosphate buffer to contain 50 U/cc. Small aliquots were stored at -25°C . The working solution, usually a 1 to 10 dilution of the stock, was stable for at least one day in an ordinary refrigerator.

Titration of Thrombin. The stop watch with a hidden face was started at the moment 0.2 cc. of thrombin solution or prothrombin activation mixture was blown into 0.8 cc. standard fibrinogen solution, contained in a test tube (i.d., 1 cm.) previously equilibrated in a 37.5°C . water bath for 20 seconds. During the first 4-second period, the pipette was put aside and the test tube was shaken. Then the tube was placed in an almost horizontal position, lowered to a 25° angle and restored to the horizontal position at the rate of 3 cycles every 5 seconds. The first visible clot formation was taken as the end point. The unit of thrombin/0.2 cc. was found by referring to a clotting time concentration curve (fig. 1) obtained by repeated tests with N.I.H. thrombin solutions. This reference curve should be prepared by each laboratory.

Collection of Blood. Ten volumes of blood was obtained from each normal individual with a syringe containing one volume of 4 per cent sodium citrate solution. The blood was centrifuged in calibrated 15 cc. graduated tubes at 1°C ., 1650 g, for 30 minutes. Therefore, the plasma hematocrit was read directly so that corrections could be made for the citrate dilution. For this particular study, 15 cc. citrated whole blood was obtained since prothrombin was determined by two methods and other

analytical tests were done. For the isolation technic alone, 10 cc. blood added to 1 cc. sodium citrate was found to be adequate.

Isolation Technic for Prothrombin. Exactly 4.0 cc. of fresh citrate plasma was pipetted into a test tube measuring 13 mm. (i.d.) by 85 mm. The tube was then placed in a -3°C . alcohol bath. When the temperature of the plasma was 0°C ., 0.72 cc. buffered alcohol was added with constant mechanical stirring. A calibrated 1 cc. tuberculin syringe and a no. 24-gauge needle were used for the alcohol addition which took approximately one-half minute. The alcohol-plasma mixture was then stirred for at least 15 additional minutes and then centrifuged at -3°C ., 1670 g, for 25 to

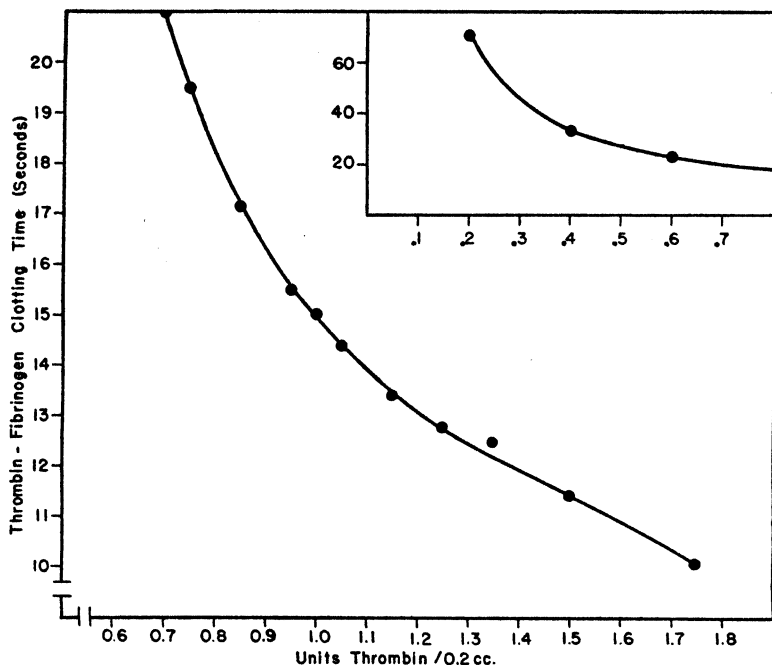


Fig. 1. CLOTTING TIME of human fibrinogen solution with varying concentrations of thrombin. Thrombin 0.2 cc. added to 0.8 cc. of 0.25 per cent fibrinogen solution previously equilibrated at 37.5°C . Citrate-phosphate buffer, $\Gamma/2$, 0.129; pH 7.2.

30 minutes. The fibrinogen fraction precipitate, which was saved for other studies (12), was firmly packed. Therefore, the test tube could be completely inverted, resulting in an almost quantitative transfer of the supernatant fluid into 40 cc. of 0°C . distilled water in a 50 cc. centrifuge tube with constant mechanical stirring and with temperature maintained at 0° to $+1^{\circ}\text{C}$. Then, 2.8 cc. 0.10 M acetic acid was added to a pH 5.05 ± 0.05 . The turbid solution was stirred for an additional 5 minutes. In 20 to 30 minutes, the now flocculent solution was centrifuged at $+1^{\circ}\text{C}$., 1670 g, for 30 minutes. The supernatant fluid was decanted off and the tube inverted for one minute at 0° to $+1^{\circ}\text{C}$. The inner wall of the tube was wiped with a clean chilled gauze without disturbing the precipitate. Immediately, 4 cc. of 0°C . citrate-phosphate buffer was added directly to the sediment and stirred with a 1 cc. sero-

logical pipette two or three times during the next 20 minutes. The resulting solution, pH 7.0-7.1, was stable for at least one day in an ordinary refrigerator and therefore no special haste to proceed to the next step was necessary. To 0.15 cc. or more of the prothrombin solution was added buffer to 1 cc. volume and 0.5 cc. thromboplastin solution, 50 U/cc. The addition of 0.5 cc. 0.025 M calcium chloride solution to the mixture at 25 to 28° C. began the activation time. Thrombin was determined on 0.2 cc. aliquots at least three times during the last 10 minutes of the 30-minute activation period. A mean of the two lowest consecutive clotting times was taken as the end point. An identical activation mixture, except that 0.2 cc. of 1 to 40 diluted fresh normal pooled plasma was substituted for an equal volume of buffer, was also made up and similarly titrated for thrombin. As control measures to make certain the absence of fibrinogen and thrombin in the prothrombin solution, 0.8 cc. and 0.2 cc. aliquots were added to 0.2 cc. (1 U) thrombin and 0.8 cc. standard fibrinogen respectively, and the clotting times taken. The prothrombin fraction remaining from each test was frozen at -25° C. or lyophilized and saved for other studies.

Thrombin Technic for Prothrombin. To 1 cc. of fresh citrated plasma was added 1 cc. (5 U) of thrombin. Clotting occurred in less than 15 seconds. In 5 to 10 minutes the clot was retracted and removed with an applicator stick. Without delay 0.25 cc. or more of the defibrinated plasma was activated as in the isolation technic with the following differences: A more concentrated thromboplastin solution (500 U/cc.) was necessary and the thrombin titrations were done every minute beginning two minutes after the calcium addition. The mean of the two lowest consecutive clotting times was taken as the end point. For a control, 0.2 cc. of the defibrinated plasma was added to 0.8 cc. of standard fibrinogen solution to test for possible thrombin. In every test the activation mixture was carefully examined for the presence of fibrin granules or clots.

Calculation of Prothrombin. The following equation was employed: $T \times \frac{1.0 \text{ cc.}}{0.2 \text{ cc.}} \times \frac{2.0 \text{ cc.}}{V} \times C = \text{U/cc. plasma}$; where T was the units thrombin obtained from figure 1; C was the correction for the citrate dilution of plasma; and, in respect to the activation mixture, 0.2 cc. was the volume titrated for thrombin, V was the volume in cc. of prothrombin solution or defibrinated plasma added, and 2.0 cc. was the total volume. Therefore, in the isolation technic the above equation was simplified: $T \times \frac{10}{V} \times C = \text{U/cc. plasma}$; and in the thrombin technic, since the plasma was diluted initially with an equal volume of thrombin, $T \times \frac{20}{V} \times C = \text{U/cc. plasma}$.

RESULTS

The clotting time data from several experiments with human Fraction I and National Institutes of Health thrombin, 12.5 U/mg., are depicted in figure 1. The resulting curve between 12 and 18 seconds generally constituted the thrombin titration standard for this study. Clotting times outside of this range were utilized for exceedingly low concentrations of prothrombin, below 10 U/cc. by the isolation

technic, and for the estimation of the most suitable volume of the solution to be added in the activation mixture.

Another series of experiments was made to demonstrate the effect of acacia on the titration of thrombin. The stock 15 per cent acacia solution was prepared according to Ware and Seegers (7). In each experiment, N.I.H. thrombin was weighed and dissolved in citrate-phosphate buffer to contain 10 U/cc. The results in table 1 show that acacia greatly accelerates the thrombin-fibrinogen reaction time and indicates that one unit as determined in this laboratory equals 1.0 N.I.H. unit or 2.46 units of Ware and Seegers (7) when human fibrinogen fraction prepared by Cohn's method (8) was used as the substrate.

Figure 2 demonstrates not only the results obtained by varying concentrations of thromboplastin but also the greater requirements of this reagent by thrombin-defibrinated plasma than by prothrombin fractions. Therefore, since a 4 per cent stock solution was the minimum concentration resulting in the lowest clotting time

TABLE 1. EFFECTS OF ACACIA AND OTHER FACTORS ON TITRATION OF THROMBIN

COMPONENTS: TITRATION MIXTURE				RESULTS			
Thrombin Solution		Human Fraction I		Units Found/Theory ¹			
Acacia Conc.	Vol.	Conc.	Vol.	Experiment			Mean
<i>per cent</i>	<i>cc.</i>	<i>%</i>	<i>cc.</i>	<i>A</i>	<i>B</i>	<i>C</i>	
0.0	0.2	0.3	0.8	0.99	1.06	0.96	1.00
0.0	0.4	1.0	0.1	0.92	1.07	0.97	0.99
2.5	0.4	1.0	0.1		2.52	2.39	2.46

¹ Three N.I.H. thrombin solutions containing 10 U/cc. were diluted so that clotting times ranged between 13 and 17 seconds on the thrombin-clotting time curve (fig. 1)

a 2.5 times stronger solution or a 10 per cent stock solution was employed in the isolation technic. Similarly, 2.5×40 per cent or a 100 per cent stock thromboplastin solution was the working reagent used in the thrombin technic.

The foregoing results are identical to those of many other experiments. The average yield of the crude thromboplastin powder was 580 mg/100 gm. human placental tissue. The average potency results from 5 experiments in units thromboplastin expressed variously were as follows: 29,000/100 gm. tissue; 50/mg. crude powder; 500/cc. stock solution; or 5000/mg. total tyrosine equivalent.

A typical experiment showing the widely diverse activation curves obtained by the thrombin and the isolation technics is depicted in figure 3. In the former, there was a critical minimal clotting time, between 5.5 and 8.5 minutes after the addition of calcium in plasma samples with normal prothrombin. With lower concentrations of prothrombin, the optimum activation time occurred more rapidly and the anti thrombin activity was even more apparent. On the other hand with the isolation technic and with all ranges of prothrombin, the clotting time dropped up to 20 minutes and then levelled off during the remainder of the 30-minute activation period.

In the experiments with the isolation technic the total tyrosine equivalents of

the whole citrated plasma and its various fractions were determined with phenol reagent (1). The Coleman Junior Spectrophotometer at $650\text{ m}\mu$ was the instrument employed. The results in table 2 clearly demonstrate chemically the efficiency of the fractionation procedure. The mean sum of the tyrosine equivalents of all the fractions was 95.6 per cent of that found in whole plasma. The prothrombin fraction represented approximately 5.5 per cent of the total tyrosine equivalent of the original sample.

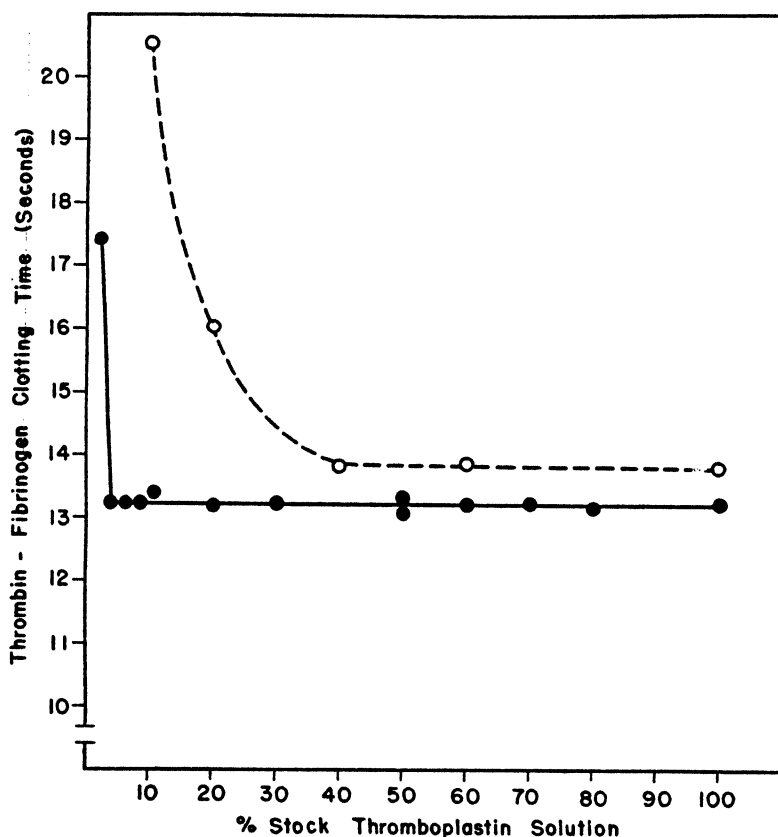


Fig. 2. VARIATION IN THROMBOPLASTIN REQUIREMENTS in two technics. $\circ - \circ$: Thrombin technic; optimum activation time, 5.5-8.5 minutes. $\bullet - \bullet$: Isolation technic; optimum activation time, 20-30 minutes. Activation: $25-28^{\circ}\text{C}$.; pH 7.2.

Another series of experiments, in duplicate, were done on 7 individual samples of plasma to evaluate the error of a single determination at varying concentrations of prothrombin. In each of plasma no. V, VI and VII, the experimental procedure was as follows: Twice-Seitz filtered plasma (20 cc/6 cm. S.T. pad) was mixed with varying volumes of the original plasma. The results by both technics are recorded in table 3. It can be concluded from the analysis of variance on data obtained on a series of duplicate tests, zero prothrombin results excluded, that the error of a single determination in the isolation technic was 2.1 U/cc. (11 degrees of freedom) in plasma

samples with prothrombin ranging between 20 and 85 U/cc ; and in the thrombin technic, 4.8 U/cc . (10 degrees of freedom) between 20 and 100 U/cc . That plasma treated simply by twice-Seitz filtration, for all practical purposes, does not contain prothrombin was determined as follows: Prothrombin fractions were obtained by the isolation technic. Maximum volumes, 0.8 cc. of this fraction, were added in the activation mixtures. After 20, 30 and 40 minutes activation, 0.2 cc. volumes of the mixtures were added to 0.8 cc. standard fibrinogen solutions. Clots and granules

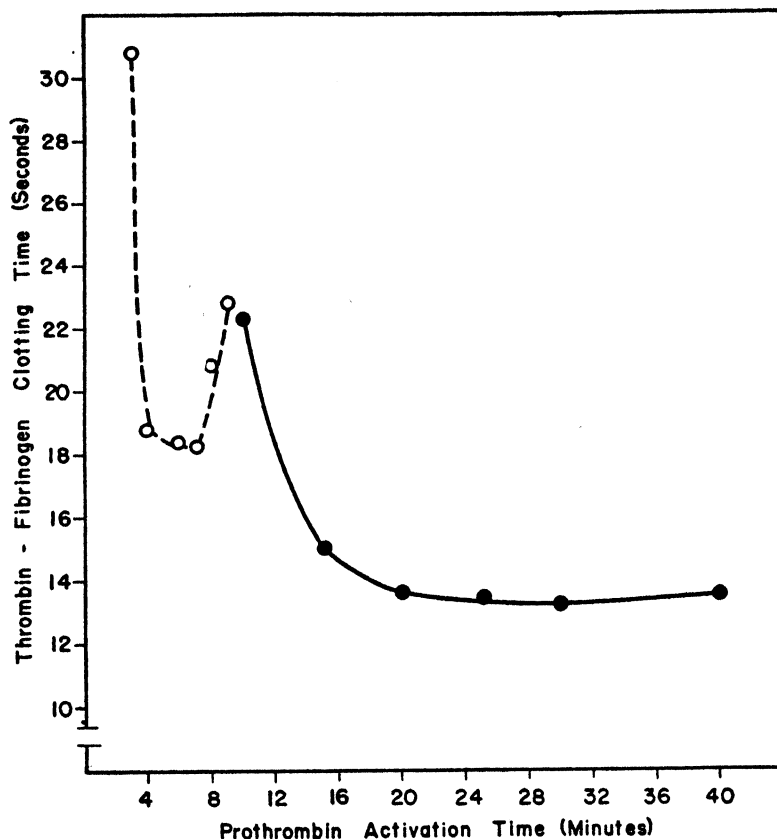


Fig. 3 VARIATIONS IN ACTIVATION PHASE of two technics. \bigcirc — \bigcirc : Thrombin technic; 0.20 cc. defibrinated plasma in activation mixture. \bullet — \bullet : Isolation technic; 0.20 cc. prothrombin fraction in activation mixture. Activation: 25–28° C.; pH 7.2.

were absent after 70 seconds, 4 hours, and even 60 hours of observation. In identical experiments with the same volume of prothrombin fractions containing, however, 2.5 U/cc ., definite clots formed in 70 seconds in contrast to no clot in 60 hours. In experiments with mixtures of filtered and non-filtered plasma, the prothrombin data in table 3 calculated for the found/expected percentages were as follows: isolation technic, 96.2, 92.2, 106.0 and 99.5 per cent; thrombin technic, 80.9, 114.0 and 84.0 per cent. The foregoing results obtained with the isolation technic were inter-

preted as additional evidence for the complete removal of prothrombin with Seitz filtration alone.

The mean prothrombin content in v/cc. of fresh plasma on 24 normal individuals ranging in age from 21 to 34 years are recorded in table 4. The correlation between the data obtained by the two technics indicated only that the results were generally lower with the isolation technic. The thrombin controls in all tests by both technics were negative in 2 minutes and in 24 hours. In the isolation technic, the fibrinogen controls showed no clots or granules after 2 minutes, when one unit of thrombin was added to 0.8 cc. of prothrombin fraction. That fibrinogen was completely removed in the defibrinated plasma was evident in the thrombin technic by the absence of fibrin clots or granules in the activation mixture. The addition of fresh normal pooled human plasma in 1 to 400 concentration in the activation mixtures did not have any effect on the results by either technic.

TABLE 2. FRACTIONATION OF PLASMA BY THE ISOLATION TECHNIC

WHOLE PLASMA OR FRACTIONS		N	TOTAL TYROSINE EQUIVALENT/CC. PLASMA		
			Mean mg.	± S.D. mg.	C.V. %
Whole Plasma		29	3.010	.280	9.14
Fractions	I	24	.311	.051	16.40
	Prothrombin	24	.167	.017	10.18
	pH 5.05 Supernatant	24	2.400	.220	9.10
	Total	24	2.878		

DISCUSSION

Any attempt to interpret the results obtained in this study requires first a critical examination of the analytical technics employed as compared with other methods for the estimation of prothrombin. The similarity of the thrombin technic and the two-stage method of Warner, Brinkhous and Smith (6) as further modified by Ware and Seegers (7) is evident in table 4. The normal result for prothrombin in fresh human plasma is reported by the latter as approximately 172 v/cc. with bovine Fraction I as the substrate in the thrombin titration; and 300 to 350 v/cc. with a more reactive fibrinogen preparation. In the present work, 110.0 v/cc. was found with the thrombin technic which differs principally in that standard fibrinogen fraction and thromboplastin of human instead of bovine origin are used and that acacia is absent. Acacia is employed in the modified two-stage technic to permit a greater dilution of plasma resulting thereby in the diminution of antithrombin activity (7). However, it was omitted here, since in all our studies on factors influencing prothrombin conversion only physiological reagents have been used. It was found that acacia alone causes a 2.46 times greater reactivity in the thrombin-fibrinogen reaction, confirming the report of Seegers and Smith (13). Therefore,

acacia is, at least, a partial explanation for the higher values for fresh normal human plasma with the modified two-stage technic of Ware and Seegers (7) than with the similar thrombin technic.

The results on the same specimens with the isolation technic averaged 82.4 u/cc. which is somewhat lower than that obtained by the thrombin technic. There are several other concrete differences in the two methods. Defibrination with thrombin is not necessary in the isolation technic, since native fibrinogen is separated out as Fraction I. It may be more appropriate to designate the thrombin technic as a three-stage procedure since the reaction of added thrombin with plasma constitutes an active coagulation process. Ware and Seegers (14) found that thrombin has variable effects on prothrombin, depending on their relative concentrations.

TABLE 3. DUPLICATE ANALYSIS BY THE ISOLATION AND THROMBIN TECHNIC ON SAMPLES CONTAINING VARYING CONCENTRATIONS OF SEITZ-FILTERED PLASMA

% SEITZ-FILTERED PLASMA IN SAMPLE	PROTHROMBIN RESULTS: U/CC. PLASMA							
	Isolation Technic				Thrombin Technic			
	a	b	Mean	Expected	a	b	Mean	Expected
0% I	49.6	51.2	50.40		72.0	71.6	71.80	
0% II	57.3	60.8	59.05		73.6	80.4	77.00	
0% III	60.5	63.2	61.85		85.4	95.6	90.50	
0% IV	58.6	55.6	57.10		83.7	88.0	85.85	
0% V	63.9	68.3	66.10		90.4	96.0	93.20	
50% V	32.3	31.7	32.00	33.05	36.6	38.8	37.70	46.60
100% V	0.0	0.0	0.00		0.0	0.0	0.00	
0% VI	69.3	70.0	69.65		89.6	104.0	96.8	
50% VI	33.3	30.7	32.00	34.83				
100% VI	0.0	0.0	0.00					
0% VII	87.5	82.1	84.80		99.7	95.0	97.35	
50% VII	46.6	43.3	44.95	42.40	54.3	54.7	55.50	48.68
75% VII	21.2	21.0	21.10	21.20	23.1	17.8	20.45	24.34
100% VII	0.0	0.0	0.00		0.0	0.0	0.00	

Another difference in the two methods lies in the nature of the prothrombin conversion. It has been shown in this study that the maximum thrombin titer in human plasma with the thrombin technic occurs at a critical point after which the enzyme rapidly disappears. This phenomenon has been previously demonstrated by Warner, Brinkhous and Smith (6). Even with the modified two-stage technic, Ware and Seegers (7) have reported the problems arising from increasing antithrombin activity in low dilutions of plasma and therefore in low prothrombin values. On the contrary, with the isolation technic, the thrombin titer reaches a maximum in approximately 20 minutes and then it is stable for the remainder of the 30-minute activation period and longer. Evidently, substances acting as antithrombin have been largely removed in this technic.

In another communication (2) it was shown that plasma proteins have a pronounced effect on the thrombin-fibrinogen reaction. However, in the isolation technic,

the low protein concentration (see table 4) would exclude this effect as an accessory factor.

The thromboplastin requirement in the thrombin technic is approximately 10

TABLE 4. CHARACTERISTICS OF FOUR TECHNIQS FOR DETERMINATION OF PROTHROMBIN

CHARACTERISTICS	ISOLATION PRESENT STUDY	THROMBIN PRESENT STUDY	MODIFIED TWO-STAGE WARE-SEEGERS (7)	ONE-STAGE QUICK (4, 5)
Removal: Plasma Fibrinogen	As Fraction I	With Thrombin	With Thrombin	None
Activation mix- ture:				
Calcium conc. M	0.0062	0.0062	0.0039	0.0083
Thromboplastin source	Human placenta	Human placenta	Bovine lung	Rabbit brain
Thromboplastin conc.	12.5 U/cc.	125.0 U/cc.	excess	excess
Albumin ¹ % conc.	0.000	0.180	0.021	1.000
Globulin ¹ % conc.	0.024	0.180	0.021	1.000
Fibrinogen ¹ % conc.	0.000	0.000	0.000	0.100
Acacia % conc. Labile factor (s) added	0.000 Human plasma	0.000 Human plasma	2.500 Bovine serum	0.000 None
Titration mixture:				
Fibrinogen source	Human Fraction I	Human Fraction I	Bovine Fraction I	Same as activation mixture
Fibrinogen % conc.	0.12-0.24	0.12-0.24	app. 0.2	
Albumin ¹ % conc.	0.000	0.036	0.017	
Globulin ¹ % conc.	0.005	0.036	0.017	
Acacia % conc.	0.000	0.000	2.000	
Standard	Human thrombin	Human thrombin	Thrombin	12.5" = 100%
Normal Values				
N	24	24	5 ²	
Mean	82.4 U/cc.	110.0 U/cc.	172.0 U/cc.	> 70% of normal
±S.D.	17.3 U/cc.	14.6 U/cc.		

¹ Based on human plasma with 3% albumin, 3% globulin, 0.3% fibrinogen, normal prothrombin.

² Dr. Walter H. Seegers, Wayne University, Detroit. Personal communication, 1949.

times greater than that in the isolation technic. It is concluded that, in the latter, any antithromboplastin activity has been largely eliminated. Experience during the last 10 years in this laboratory has shown that the standardization of thrombo-

plastin reagent is the most important factor influencing the accuracy of prothrombin determinations with any technic. Therefore, in the present study, this reagent is titrated with prothrombin solutions of known activity and a 2.5 times excess employed in the two technics. The latter was arbitrarily chosen as sufficient for an adequate margin of safety in analysis of normal plasma: It is also an application of the concept of a controlled excess of a component in the study of a biological system. The simple procedure described here resulted in human thromboplastin preparations which satisfied the criteria established for the present study. These included stability, standardization, high yield, moderate activity and, most important, absence of other coagulation factors. The last was achieved by repeated high gravity centrifugation and by heating at 56° C., and confirmed by control tests. The ready availability of fresh human placenta in comparison to brain or lung of any species requires no further comment.

One of the most important of recent developments in the field of coagulation has been the discovery of the new Factor V of Owren (15), accelerator factor of Fantl and Nance (16), Ac-globulin of Ware, Guest and Seegers (17), and the labile or plasmatic co-factor of Quick (18) and Honoratio (19). This factor was considered in this study by the introduction of fresh diluted plasma into prothrombin conversion mixtures. However, no significant change in the conversion rate or the strength of prothrombin could be observed over the controls in any of the determinations in fresh normal plasma by either the isolation or thrombin technics. Therefore, it is assumed that this new factor existed in adequate amounts. Murphy and Seegers (20) have shown that fresh normal human plasma contains very little Ac-globulin and that almost identical prothrombin results are obtained with or without the added factor.

SUMMARY

An homologous isolation technic for the determination of prothrombin on 4 cc. samples of plasma is described. It is based on the low temperature, low ionic strength alcohol removal of Fraction I (Cohn) and the subsequent isoelectric precipitation of a stable thrombin- and fibrinogen-free prothrombin fraction. The latter is activated with an optimum concentration of calcium and a known excess of placental thromboplastin. The resulting thrombin is titrated with a fibrinogen solution. A standard thrombin preparation is the final reference of potency. Methods for the preparation and standardization of fibrinogen and thromboplastin are outlined. Results from experiments to evaluate the error of a single determination, the recovery of prothrombin, the efficiency of the fractionation, and the reaction with thromboplastin are presented. The average value obtained on 24 normal individuals ranging in age from 21 to 34 years is 82.4 U prothrombin/cc. plasma.

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EFFECT OF BLOOD PLATELETS ON PROTHROMBIN UTILIZATION OF DOG AND HUMAN PLASMAS¹

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IT HAS been demonstrated, qualitatively, that the rate of blood coagulation is related to the presence of blood platelets. This relationship has been known to exist in bird blood since the investigations of Delezenne (1). In mammalian blood, however, conflicting data and opinions regarding the rôle of platelets in coagulation have been published during the past half-century. Only in the past decade has it been clearly demonstrated that platelets play an important rôle in the clotting process. In 1939 one of us (2) showed that prothrombin is slowly utilized in human blood if it is immediately centrifuged to reduce the number of platelets present during clotting. More recently, Jaques and co-workers (3) showed that platelet-poor plasmas have a prolonged clotting time. In their experiments, platelet alterations during the preparation of plasma were prevented by use of silicone-treated equipment. Later work demonstrated that plasma, carefully prepared with a silicone technique to minimize or prevent platelet rupture, clots very slowly or not at all when freed of platelets. Incoagulable platelet-free plasma has been obtained from both human and dog blood (4, 5).

The purpose of this investigation was to determine the platelet levels at which impairment of the clotting process appears, and to compare the platelet requirements of human and dog blood.

METHODS

The method for obtaining the blood and plasma was as follows: syringes, needles (16-18 gauge) and glassware were treated with a 10 per cent solution of a methychlorosilane (General Electric Dri-Film) in benzene. Blood was obtained from the external jugular vein in the dog and from the cephalic vein in the human. No samples were used in which difficulties were encountered in the venepuncture or in blood withdrawal, or in which there was evidence of lipemia. The blood was collected in a series of three syringes. In the first syringe, 4 to 6 ml. of blood were obtained for determination of the hematocrit. In the second syringe, 4 ml. of blood were obtained for platelet counts. And in the third, 45 to 50 ml. of blood were obtained without anticoagulant for the clotting studies. Blood from the last syringe was cooled rapidly in silicone-treated tubes in an ice bath. To reduce the number of platelets to the desired level, the blood was centrifuged for varying periods of time in angle centrifuges. For dog samples, a centrifugal force of about 1450 g for 2 to 15 minutes was used. For human samples the centrifugal force was about 1650 g for the same periods of time. To obtain platelet-poor plasmas containing less than 5,000 to 10,000 platelets per cu. mm., a centrifugal force of about 15,000 g was used. Collection and centrifugation of the blood were carried out in a constant temperature room (2° C.).

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Two measures of the rate of clotting were used, the clotting time and the prothrombin utilization rate. In both tests, timing of the clotting process was started when the blood or plasma was placed in ordinary glass tubes. For the clotting time determinations, 1 ml. of whole blood or 0.5 ml. of native plasma was transferred with a silicone-treated needle and syringe to each of two dry 10 x 75 mm. glass tubes. For the determination of the rate of prothrombin utilization (2), blood or plasma was transferred in a similar manner to a series of tubes containing 0.15 ml. of imidazole buffer at pH 7.3 (6). At frequent intervals during the next 50 to 60 minutes, the contents of each of two tubes were mixed with 0.12 to 0.16 ml. of 3.2 per cent sodium citrate solution to stop the conversion of prothrombin to thrombin. Prothrombin determinations, using the two-stage method of Warner, Brinkhous and Smith (7, 8), were made promptly on the plasma or serum. The above procedures were carried out at 27° to 28° C.

Platelet counts on the whole blood were performed by a modification of Nygaard's method (9), using 4 parts of 3.2 per cent sodium citrate to one part of whole blood. After sedimentation of dog blood for 15 minutes, and of human blood for 30 minutes, supernatant plasma was transferred to a counting chamber and the platelets counted. Direct platelet counts were made on the native

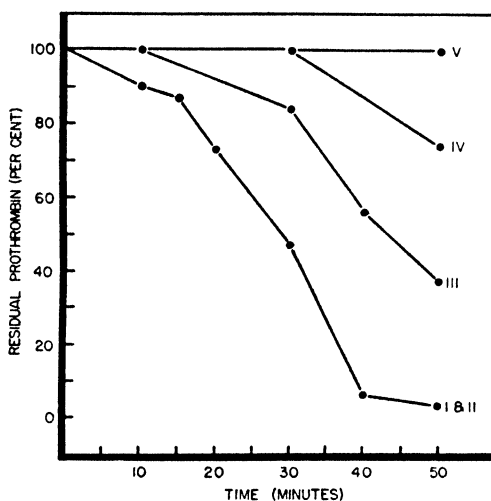


Fig. 1

plasma specimens obtained by centrifugation. One of us (J. A. B.) performed all the counts, which were done in duplicate or triplicate.

RESULTS

Sixteen healthy adult dogs and 9 healthy adult human subjects, selected without regard to sex, were used. In all, 48 experiments were performed on dog blood and 32 experiments on human blood. The mean number of platelets for dogs was 383,000/cu. mm. of whole blood, or 658,000/cu. mm. of plasma, with a standard deviation of 82,000. The mean value for the human subjects was 285,000/cu. mm. of whole blood, or 549,000/cu. mm. of plasma, with a standard deviation of 59,000.

The results of one group of experiments with dog blood are given in figure 1 and table 1. This group exemplifies the experimental procedure followed throughout this work. The residual prothrombin content of the whole blood and plasma samples during the course of clotting is shown in the figure. It is seen that in the plasma with 58 per cent of the original number of platelets (curve II), the prothrombin disappeared

at the same rate as in the whole blood (curve I). Plasmas containing 22 per cent of the original number of platelets or less (curves III and IV) showed a considerable retardation of the rate at which prothrombin disappeared. The plasma in which only a few platelets remained showed no loss of prothrombin during the experimental period (curve V).

To obtain a numerical expression of the extent of the retardation of clotting, a prothrombin utilization index was devised. This index represents the ratio of the amount of prothrombin utilized in the plasma to the amount utilized in the normal whole blood. The method of calculation of the index is shown in table. 1. The interpolated points on the whole blood curve (fig. 1) at which 75, 50, and 25 per cent of the original prothrombin remained in the serum were selected. The times on the

TABLE 1. EFFECT OF PLATELET CONTENT OF PLASMA ON CLOTTING TIME AND PROTHROMBIN UTILIZATION INDEX

CURVE NO. (see fig. 1)	SPECIMEN	PLATELETS		CLOTTING TIME	PROTHROMBIN UTILIZED			PROTHROMBIN UTILIZATION INDEX
		Per cu. mm. of plasma $\times 10^3$	No. relative to whole blood		Minutes			
					19	28	35	
I	Whole blood	650	%	min.	%	%	%	
II	Plasma (2 min. centrifugation)	380	100	9	25	50	75	$\frac{150}{150}$ or 1.0
III	Plasma (3 min. centrifugation)	140	58	10	25	50	75	$\frac{54}{150}$ or 0.36
IV	Plasma (4½ min. centrifugation)	86	22	14	8	15	31	$\frac{8}{150}$ or 0.05
V	Plasma (150 min. centrifugation)	<0.3	13	27	0	0	8	$\frac{0}{150}$ or 0.00
			<1	>35	0	0	0	

abscissa were found to be 19, 28, and 35 minutes respectively. Then the corresponding prothrombin values on the plasma curves were obtained. The average ratio of the prothrombin *utilized* at the indicated times was then determined. Values of less than 1.0 indicate that clotting is impaired—the slower the clotting the lower the value of the prothrombin utilization index.

Figures 2 and 3 show the relationship between the platelet content of plasma and the rate of clotting. In the dog plasmas, it will be observed that an impairment of clotting occurred when the number of platelets was reduced below about 35 per cent of the number present in whole blood, or about 230,000 platelets per cu. mm. of plasma (fig. 2). In the human plasmas, on the other hand, no impairment of clotting was evident until the number of platelets was below about 25 per cent of the original value, or about 135,000 platelets per cu. mm. of plasma (fig. 3). When the platelets were reduced to a range of 5 to 15 per cent in dog plasma, frequently no prothrombin disappeared during the period of observation. In these cases, the prothrombin utilization index was 0. In human plasma, considerably greater reduction in the number of

platelets was required to prevent utilization of prothrombin in the experimental period.

The clotting time was a less sensitive index of changes in the course of clotting than was the prothrombin utilization rate. In human plasma, even with a reduction of platelet levels to 5 per cent of the original whole blood values, no prolongation of the clotting time was noted. Only when the platelets were at a level of about one per cent or lower, was a consistent delay in clotting time observed. In dog plasma, on the other hand, a prolongation of the clotting time was observed regularly when the platelet levels were reduced below about 15 per cent of the original values. With extremely low platelet counts in either type of plasmas, clotting generally did not occur during the 50 to 60 minute period of observation.

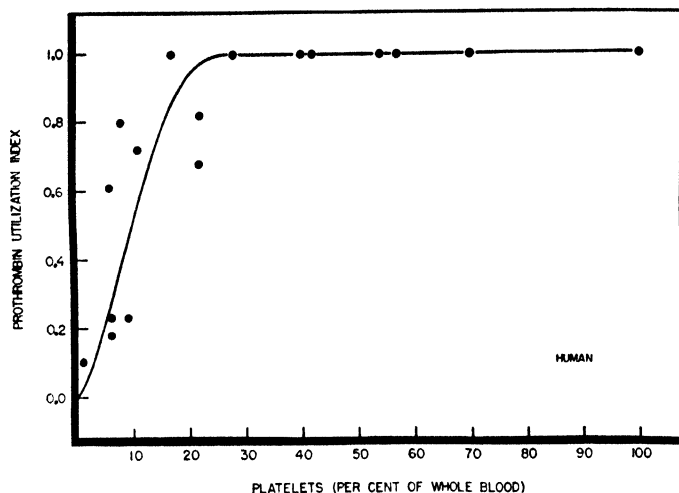


Fig. 2

DISCUSSION

These results emphasize the need for platelets in clotting and indicate that normally platelets are present in numbers considerably in excess of minimal requirements for normal clotting. Roughly, there is a three-fold factor of safety in dog blood, and a four- or five-fold safety factor in human blood. The difference between the platelet needs of dog and human blood is more striking if considered in terms of platelet volume. Normally, according to Van Allen (10), the average volume percentage of platelets for dog blood is 1.04, for human blood 0.49. Thus, the minimal platelet volumes required for normal clotting would be about 0.36 ml/100 ml. of dog blood and about 0.12 ml/100 ml. of human blood. By volume, then, dog blood requires about three times as much platelet material as human blood, compared to about 1.7 times as many if considered in terms of platelet numbers.

The fundamental reason for the greater need for platelets in dog blood is not clear. Whether there are qualitative or quantitative differences in the platelet coagulant factors in the two species is not known. Earlier work has shown that the

plasma factor deficient in hemophilic blood is necessary for platelet utilization (4). It may be that the quantity of the anti-hemophilic principle in plasma determines the extent to which platelets are utilized in the clotting process. At any rate, our data indicate that normally the number of platelets is not the factor which determines the rate of coagulation, and only when their numbers are greatly reduced do they limit the speed of clotting.

A comparison of our findings on normal human plasma with results obtained on blood from patients with thrombocytopenia is of interest. In this disease, the clotting time is nearly always normal. Our data are in accord with this fact, since platelets rarely reach the low levels that would be required for a prolonged clotting time. Soulier (11) has used a modified two-stage method for the determination of residual prothrombin in the serum of a group of thrombocytopenic patients. Seven-

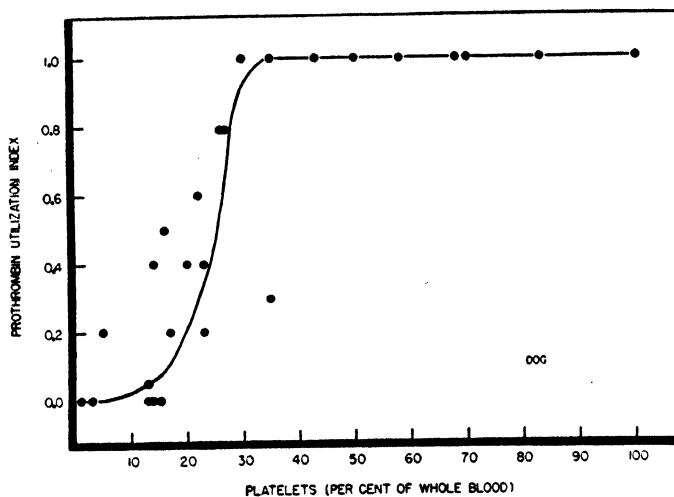


Fig. 3

teen of his patients showed a retardation in the clotting process, as judged by high serum prothrombin levels. All of these patients had platelet counts below 81,000/cu. mm. This is in good agreement with our data, which indicate that the average critical level of platelets is equivalent to a value of about 71,000/cu. mm. of whole blood. On the other hand, there is less evident agreement between our data and those of Conley, Hartmann and Morse (12) and of Quick, Shanberge and Stefanini (13). These authors studied both blood from thrombocytopenia patients and platelet-poor normal plasmas. From their work, it would appear that only a moderate reduction in the number of platelets is sufficient to cause a delay in clotting. They used a one-stage technique for determination of serum prothrombin. This procedure results in erroneously high serum prothrombin values, due apparently to the fact that thrombin is formed more rapidly from prothrombin in serum than it is from prothrombin in plasma. De Vries, Alexander and Goldstein (14) have suggested that the difference in prothrombin convertibility in plasma and serum, as observed in the one-stage method, is due to the elaboration in serum of an accelerator of prothrom-

bin conversion. This factor, as well as other differences in technique, may account for these apparently divergent results.

SUMMARY

The platelet requirements for clotting in dog and human blood have been compared. The rate of prothrombin utilization was used to indicate the clotting capacity of plasmas containing varied numbers of platelets. The results indicate that in both plasmas platelets are present in great excess over minimal requirements, and that below critical platelet levels, clotting is impaired. Dog plasma requires more platelets for a normal clotting rate than does human plasma. In terms of platelet count, the requirements of dog plasma are about 1.7 times greater than they are in human plasma; in terms of platelet volume, the requirements are about 3 times greater.

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EVOLUTION OF A PROTHROMBIN CONVERSION ACCELERATOR IN STORED HUMAN PLASMA AND PROTHROMBIN FRACTIONS¹

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THE prothrombic activity of plasma, as measured by the one-stage method, decreases as plasma ages (1-3) because of deterioration of 'labile factor' necessary for the rapid conversion of prothrombin to thrombin by thromboplastin plus calcium. When, however, determinations are made by a modified procedure in which the test plasma is first diluted with *prothrombin-free fresh* plasma, the aged plasma shows *increased* prothrombic activity (3). Similar observations have been recorded by others (4-6).

From the data presented below it appears that the phenomenon is referable to the evolution during storage of a factor which can accelerate prothrombin conversion. This substance, similar to or identical with a prothrombin conversion accelerator which is elaborated during coagulation (7), can be separated from stored plasma by adsorbing it with BaSO₄ from which it can be eluted by sodium citrate.

METHODS

Oxalated human plasma (1 volume of 0.1M sodium oxalate to 9 volumes of blood), prepared from blood centrifuged at 2000 rpm for 10 minutes, was stored at 3 to 5° C. At intervals prothrombic activity was determined both by the one-stage method of Quick (8), and by the modification of Rosenfield and Tuft (9) in which prothrombin-free plasma is used as diluent. The latter was prepared from oxalated plasma pooled from at least 5 normal subjects. In both procedures commercial thromboplastin (Difco) was used.

Fresh and stored plasma samples were also treated in the following manner: powdered BaSO₄ (C.P.) was added, the mixtures were shaken and kept at 37° C. for 15 minutes during which time they were frequently agitated, and then centrifuged at 3000 rpm for 30 minutes. The supernatant was separated; the BaSO₄ was washed twice with an equivalent volume of sodium acetate buffer (0.02M, pH 5.2) and eluted (once or twice) with sodium citrate solution (5% in physiological saline) which totaled in volume that of the original plasma.

The prothrombic activities of the supernatants and eluates were determined in the usual manner after diluting with fresh prothrombin-free normal plasma. The prothrombin conversion accelerator was measured by mixing these fractions with equal volumes of fresh whole plasma and computing the difference between the observed prothrombic activities of the mixtures and the sum of the activities of the components determined separately, employing the same dilution technique with prothrombin-free plasma.

RESULTS

The data, recorded in figure 1, confirm the fact that as plasma ages its prothrombic activity, as measured on undiluted whole plasma, decreases progressively.

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However, when determinations are made using fresh prothrombin-free plasma as diluent, prothrombic activity increases during storage at refrigerator temperature until the aged plasma becomes approximately two or three times as active as the original unstored plasma. Thereafter, the activity slowly declines. The 'hyperactivity' is not demonstrable in plasma which ages at room or body temperatures.

The interval of storage required for the development of increased prothrombic activity varies widely. In some instances the change becomes evident within 24 or 48 hours; in others, two weeks or more are necessary (table 1). When citrate (one part of 2.5 % sodium citrate solution to 9 parts of blood) is used instead of oxalate as anticoagulant the appearance of hyperactivity is delayed. Also, as has been observed previously by others (10) and by us (3), the decrease in whole plasma prothrombic activity is retarded.

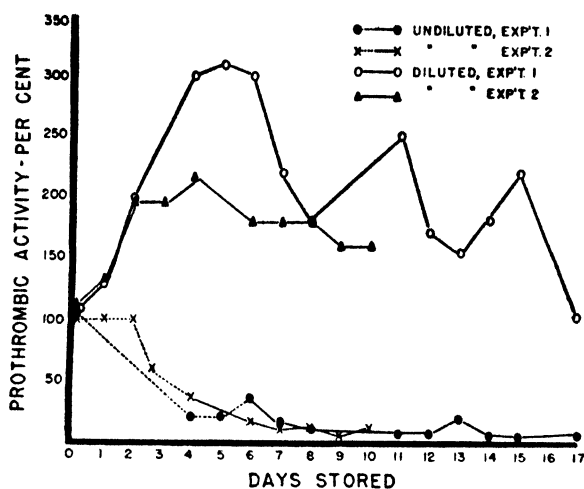


Fig. 1. PROTHROMBIC ACTIVITY OF OXALATED HUMAN PLASMA stored at 4°-5°. C Determinations made at intervals on the same plasma by the orthodox one-stage procedure on whole plasma (undiluted) and by the dilution technique (diluted) employing fresh BaSO₄ plasma as diluent.

Prothrombin can be adsorbed from plasma by BaSO₄ (9). We have found that it can be eluted from this adsorbing agent by sodium citrate. The 'hyperactive' prothrombin of stored plasma behaves similarly. When plasma is adsorbed at intervals during storage with 25 mg. BaSO₄ per cc. and the adsorbate eluted with sodium citrate, the prothrombic activities of the eluates parallel those of the unadsorbed parent plasma (fig. 2). The supernatants from the BaSO₄ adsorption show little or no activity (fig. 2).

An eluate from the BaSO₄ adsorbate obtained from a 'hyperactive' (166% of normal prothrombic activity) stored plasma exhibited only 10 per cent prothrombic activity and contained 31.1 micrograms of nitrogen derived from 1 cc. of plasma (table 2). However, when it was mixed with fresh plasma, the observed activity of the mixture was almost threefold the sum of the activities of the components. Separation thus of a plasma fraction, poor in prothrombin, yet capable of enhancing the prothrombic activity of fresh plasma to which it was added, indicates that the

'hyperreactivity' which develops in stored plasma is due to the evolution of a prothrombin conversion accelerator and not to deterioration of an inhibitor.

Prothrombin-rich fractions derived from fresh plasma by BaSO₄ adsorption and citrate elution also show increasing activity during storage (table 3). In contrast to some of the parent plasmas which were relatively slow in becoming hyperreactive the activity of the eluates increased within 24 hours (table 1). It should be noted that determinations on the eluates and the parent plasmas were made side by side, after both were adjusted to contain equal amounts of oxalate and citrate. The supernatants, very low in activity originally, remained unchanged. This indicated that the precursor of the accelerator which evolves during storage is adsorbed along with the prothrombin.

TABLE 1. PROTHROMBIC ACTIVITY OF PLASMA AND PLASMA FRACTIONS DURING STORAGE AT 3-5° C.

PLASMA FROM SUBJECT	TIME REQUIRED FOR HYPERREACTIVITY TO APPEAR, DAYS		PROTHROMBIC ACTIVITY ¹			
	Whole Plasma	Eluate from BaSO ₄ Adsorption	Whole Plasma		Eluate from BaSO ₄ Adsorption	
			Initial	Maximal ²	Initial	Maximal
A	1		80	200 (4)		
B	1		105	190 (4)		
C	10		75	190 (15)		
D	4		66	230 (7)		
ABCD (pool)	1		78	230 (7)		
E	15		59	140 (15)		
E ₂ ³	>7	1	85		49	79
F	10		71	130 (15)		
F ₂ ³	>8	1	85		68	108
G	4		63	170 (7)		
G ₂ ³	5	1	75	140 (6)	75	105

¹ Percentage of normal.

² Figures in parentheses denote the day of storage at which maximal activity was first observed.

³ Plasma obtained from subjects E, F and G on other days.

It is possible that an accelerator might not be evident in the aged 'hypoprothrombinemic' plasma because its initial prothrombin content might be so small that an enhancement in activity might not be striking. Under such circumstances, addition of prothrombin in the form of fresh normal plasma or prothrombin-rich fractions should result in a greater prothrombic activity than would be expected on the basis of the added prothrombin alone. When prothrombin was thus added to the deprothrombinated stored plasma, no enhancement could be observed; the activity of these mixtures was equal to the sum of the activities of the components determined separately. When the prothrombin was only partially removed by adsorbing with smaller amounts of BaSO₄, the 'hypoprothrombinemic' plasma (30%) of normal showed some rise in activity during storage (fig. 3) although less than the whole parent plasma.

Dicumarol-induced hypoprothrombinemia was also studied. Three patients, treated with this drug for impending or actual myocardial infarction, had between 5

and 12 per cent prothrombin. During storage the plasmas of two showed some increase in activity, the earliest change appearing after 14 days (table 4). However, when prothrombin was added in the manner described above, little, if any, further acceleration was demonstrable.

Thus, plasma rendered markedly hypoprothrombinemic either by BaSO_4 adsorption or by dicumarol administration does not become strikingly 'hyperreactive' during storage or develop the ability to accelerate the conversion of added prothrombin. Similar results were obtained in a patient with hypoprothrombinemia due to severe hepatic cirrhosis (table 4). It is not clear whether this is referable to reduction of prothrombin per se or to simultaneous inadequacy of a non-prothrombin precursor of the accelerator. There is evidence that dicumarol lowers, besides prothrombin, a substance which affects prothrombin conversion (11, 12). Also, the patient with liver disease seemed to be deficient not only in prothrombin but also in

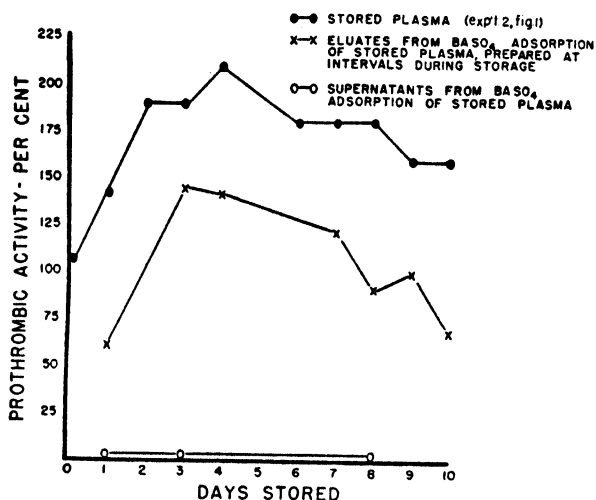


Fig. 2. FRACTIONATION OF NORMAL OXALATED PLASMA at intervals during storage: separation of 'hyperreactive' prothrombin by adsorption with BaSO_4 and elution with sodium citrate solution.

plasma factors important in prothrombin conversion since the prothrombic activity of her whole plasma (14% of normal) was far less than that (33%) obtained with the dilution technique employing *prothrombin-free normal* plasma. Observations were also made on a 5-week-old patient with congenital hypoprothrombinemia² whose plasma prothrombic activity was 1 to 2 per cent of normal. During 6 days' storage it did not change appreciably nor did any accelerator evolve. Unfortunately, further observations could not be made since the patient died.

Platelets are not required for evolution of the accelerator. Normal oxalated plasma was rendered platelet-'free'³ by centrifugation for 15 minutes at 15,000 rpm in a Type 2 international centrifuge with multi-speed attachment, kept in a constant

² We are grateful to Dr. Charles A. Janeway, Physician-in-Chief of the Children's Hospital, Boston, for his cooperation in making this subject available for study. Details will be reported in a separate communication.

³ Counts made on the centrifuged plasma revealed 10,000 platelets or 'platelet bodies' per cu. mm. of plasma.

temperature room at 6°C. During storage the plasma developed increased prothrombic activity, parallel with the non-centrifuged plasma (fig. 4). The blood had been handled throughout in apparatus coated with silicone⁴ until the plasmas were placed in ordinary glass for storage. Also, the accelerator could be separated from the aged platelet-free plasma by BaSO₄ adsorption and subsequent elution. Furthermore, plasma from a patient with thrombocytopenic purpura secondary to acute leukemia (platelet count = 30,000 per cu. mm. of blood) became hyperreactive during storage. That the platelets cannot, therefore, be implicated in evolution of the accelerator is in contrast to the interpretations of others (6, 14) who attributed the prothrombic hyperreactivity of stored plasma to the products of platelet lysis.

The question also arises whether exposure to a 'foreign' surface is required. Plasma derived from blood taken with siliconized apparatus and stored in siliconized

TABLE 2. DEMONSTRATION OF PROTHROMBIN CONVERSION ACCELERATOR IN A PLASMA FRACTION FROM "HYPERREACTIVE" STORED PLASMA

PROTHROMBIN MIXTURE CONTAINING:				PROTHROMBIN		
Stored Plasma (S.P.), Eluate (E) or Supernate (S)	Whole Fresh Plasma	Saline	Diluent: Proth.-Free Fresh Plasma	Time	Activity ¹ Found	Activity Expected
<i>parts</i>	<i>parts</i>	<i>parts</i>	<i>parts</i>	<i>sec.</i>	<i>%</i>	<i>%</i>
S.P.—1	0	0	9	20.9	166	
E. ² —3	0	0	7	69.0	10	
E. ² —1	1	0	18	21.6	154	53
S.—1	0	0	4	33.8	34	
S.—1	1	0	18	30.3	76	65
0	1	1	18	41.8	96	

Pooled oxalated plasma stored at 4°–5°C. for 14 days, then adsorbed with 25 mg. BaSO₄/cc. The BaSO₄ was then eluted with 5% sodium citrate in 0.9% saline solution; final volume was that of original plasma.

¹ Corrected for dilution of prothrombin mixture with prothrombin-free fresh plasma.

² Eluate contained 31.1 µg. N/cc.

vessels becomes equally hyperreactive at the same time as plasma from the same individual handled throughout in ordinary glass.

The prothrombin accelerator evolves also in stored hemophilic plasma. Of 5 hemophiliacs studied the plasmas from 2 showed increasing activity within 24 to 48 hours; in the others, a much longer time was required before any change was demonstrable. These phenomena could not be correlated with the clotting time of whole blood nor were they affected by accelerating coagulation with intravenous infusions of normal plasma (150–180 cc.) before obtaining the hemophilic plasma for storage.

On the theory that small amounts of thrombin, slowly evolved from prothrombin during storage, might be required for elaboration of the accelerator from an inert precursor (see later in discussion regarding conversion of plasma Ac-globulin to the serum type by thrombin) 0.1 and 0.2 units of thrombin (Parke, Davis topical thrombin) were added to 2.0 cc. of fresh chilled oxalated hemophilic plasma which alone showed delayed increase in prothrombic activity upon aging. The addition of throm-

⁴ General Electric Dry Film #9987 used according to the technique of Jacques *et al.* (13).

bin did not accelerate the appearance of hyperreactivity. Small fibrin shreds and thin clots were observed in 24 hours, but the amount of fibrinogen thus removed had no demonstrable influence on the prothrombin time.

DISCUSSION

Changes in plasma prothrombic activity during storage have been studied by many workers. Non-uniformity of methods has led to divergent results and interpretations. Clearly the activity, as determined on whole plasma by the one-stage technique, declines after the first few days of storage, due to deterioration of a labile component which is present in fresh plasma (3).

When determinations are made on mixtures of the stored, with prothrombin-free fresh, plasma, the activity is found to increase progressively until, at its height, it may be two or three times the initial value. Enhanced reactivity of fibrinogen to

TABLE 3. PROTHROMBIC ACTIVITY OF PLASMA FRACTION DURING STORAGE AT 4-5°C.

DAYS STORED	PROTHROMBIC ACTIVITY—PER CENT ¹			
	15 mg. BaSO ₄ Adsorption		25 mg. BaSO ₄ Adsorption	
	<i>Eluate</i>	<i>Supernatant</i>	<i>Eluate</i>	<i>Supernatant</i>
0	70	10.0	120	0
1	140	10.5	140	0
2	115	9.0	155	0
4	165	9.0	200	0
7	128	11.4	170	0
8	126	10.8	190	0
9	170	8.0	180	0
10	158	—	158	—

Aliquots of oxalated plasma from one subject adsorbed with 15 mg. and 25 mg. BaSO₄ respectively. Prothrombic activities determined on supernatant plasmas, and on eluates obtained from the BaSO₄ by elution with 5% sodium citrate in saline.

¹ On basis of normal plasma containing 100% prothrombic activity

thrombin, one of the proposed explanations for this phenomenon (4), can be readily dismissed: the hyperreactivity of aged plasma is measured in a mixture in which 90 per cent or more of the clottable fibrinogen is provided by fresh prothrombin-free plasma. Furthermore, plasma fractions devoid of fibrinogen become hyperreactive during storage. Finally, we have found (15) that stored plasma becomes less clottable by standard solutions of thrombin.

Increasing prothrombic activity may reflect deterioration of an inhibitor such as an antiprothrombin, antithrombin or antithromboplastin. This explanation is also untenable since a prothrombin conversion accelerator can be separated from hyperreactive stored plasma.

Banfi *et al.* (6) attribute the hyperreactivity of stored plasma to a prothrombin 'sensitization,' whereby, under the influence of deteriorating platelets, prothrombin undergoes a molecular alteration yielding a product more rapidly convertible to thrombin by thromboplastin plus calcium. This interpretation, which resembles Bordet's (16) concept of proserozyme (less active prothrombin) being converted

during coagulation to serozyme (more active prothrombin), can be excluded also by the fraction obtained from stored plasma, which was itself very low in prothrombic activity but, nevertheless, could markedly accelerate thrombin evolution from added prothrombin.

The prothrombic 'hyperreactivity' of stored plasma is best explained by slow elaboration of a factor which activates, accelerates or otherwise acts as an ancillary agent in the evolution of thrombin in the presence of thromboplastin plus calcium. Formation of similar substances has also been observed during blood coagulation (17, 18). Ware *et al.* (17) have reported on *serum* Ac-globulin which arises, under the influence of small amounts of thrombin, from a relatively inert precursor, *plasma* Ac-globulin. During storage slow conversion of prothrombin to thrombin may occur despite the presence of anticoagulant, thus providing the conditions necessary for transforming plasma Ac-globulin into the serum type. It will be recalled, however,

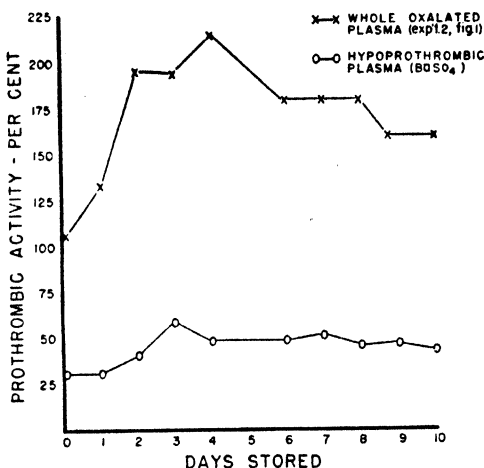


Fig. 3. PROTHROMBIC ACTIVITY, during storage at 4°-5° C., of BaSO₄ adsorption induced 'hypo-prothrombic' plasma and the parent plasma.

that the addition of small amounts of thrombin to hemophilic plasma failed to hasten the appearance of increased prothrombic activity.

Milstone (19) described a prothrombin convertor, thrombokinase, which evolves from an inert precursor, prothrombokinase, during the first stage of coagulation. Calcium is required for the transformation; whether it can proceed in oxalated plasma is unknown. It is unlikely that the accelerator which evolves in stored plasma is thrombokinase because prothrombokinase is not adsorbed by BaSO₄ (19) whereas the precursor of the accelerator which evolves in stored plasma is adsorbed.

A prothrombin conversion accelerator which arises during coagulation has also been demonstrated in human serum by de Vries *et al.* (7). It is relatively stable, in contrast to the extreme lability of Ac-globulin in human serum (10), can be adsorbed quantitatively by BaSO₄ or BaCO₃ and eluted by citrate solutions (20), and it is greatly reduced in serum from hypoprothrombinemic blood.⁵ Its remarkable re-

⁵ In congenital hypoprothrombinemia, as well as that induced by dicumarol (21).

semblance in these respects to the accelerator which evolves in stored plasma suggests that as plasma ages very slow 'coagulation' occurs during which the 'serum' prothrombin conversion accelerator evolves. This would explain the deposition of fibrin clots during storage despite the presence of anticoagulant.

That the accelerator cannot be detected by determinations on *whole* stored plasma⁶ suggests that labile factor or some other plasma component similarly labile is necessary for its activity. During the first several weeks of storage, prothrombic activity, as measured by the orthodox one-stage technique, is the resultant of prothrombin concentration, unaltered labile factor and evolved prothrombin conversion accelerator. This must be considered in assays of labile factor based upon restoring prothrombic activity to stored plasma by adding fresh plasma or plasma fractions (5).

Progressive increase in the activity of aging prothrombin-rich plasma derivatives brings to mind the report of Ware and Seegers (22) on 'regeneration' of purified bovine prothrombin in the presence of thrombin. The question arises whether their observations are referable to evolution of a factor which favorably affects the yield as well as the velocity of thrombin formation.

TABLE 4. PROTHROMBIC ACTIVITY OF STORED HYPOPROTHROMBINEMIC OXALATED PLASMA

SUBJECT	Days Stored	0	1	PROTHROMBIC ACTIVITY—PER CENT					17	21	24
				3	7	14					
				<i>Dicumarolized subjects</i>							
Mrs. D.	9	5	6	12	22				18		
Mrs. B.	12	16	9	27	20				22		
Mr. S.	12	15	7	12	13				14		
				<i>Subject with cirrhosis of liver</i>							
Mrs. H.	33		35	36	28				44	43	35

Changes in activity of prothrombin-rich fractions deserve careful consideration in the purification of prothrombin when guided by the one-stage technique. Also, prothrombin assays by the two-stage procedure must be viewed with caution until one can be sure that an accelerator evolving in a prothrombin fraction does not increase the yield of thrombin. In this connection there should be mentioned the recent observations of Lewis and Ferguson (23) that no limit is reached in the amount of thrombin obtained from a given quantity of purified prothrombin to which increasing amounts of Ac-globulin are added.

Little can be said regarding the precursor of the accelerator. Platelets can be excluded by the experiments on thrombocytopenic plasma and on normal plasma stored in siliconized vessels. Our findings on plasma rendered prothrombin-deficient by dicumarol or by adsorption with BaSO₄ suggest that the plasma prothrombin concentration must be at least 30 per cent of normal before substantial amounts of the accelerator can evolve. However, this is probably not the sole requisite, as indicated by observations on the cirrhotic patient whose plasma, although it contained 30 to 40 per cent prothrombin, failed to become hyperreactive. This may have been related

⁶ Schilling *et al.* (13) report 'hyperactivity' of stored citrated plasma within the first 5 days of storage. The increases in activity, 10-12 per cent, is only suggestive in view of limitations in the method they employed.

to the inadequacy of non-prothrombin factors important in the evolution of thrombin from prothrombin,⁷ which was observed in this subject.

Thus, two important changes in plasma clotting components have been demonstrated during storage: elaboration of an accelerator which favorably affects the velocity of prothrombin conversion, and deterioration of labile factor, which adversely influences this reaction. The question arises whether these phenomena are related. At first glance it appears that they are not since prothrombic hyperreactivity may appear in some instances before any deterioration of labile factor is detectable from determinations of the prothrombin time on whole plasma. However, as has already been mentioned the prothrombin time of plasma, at any point during storage, reflects the concentration of prothrombin, labile factor and the prothrombin conversion accelerator which evolves. Therefore, normal prothrombin time on the second, third or fourth day of storage does not necessarily exclude some deterioration of labile factor. Some loss in this component may have occurred, yet because of

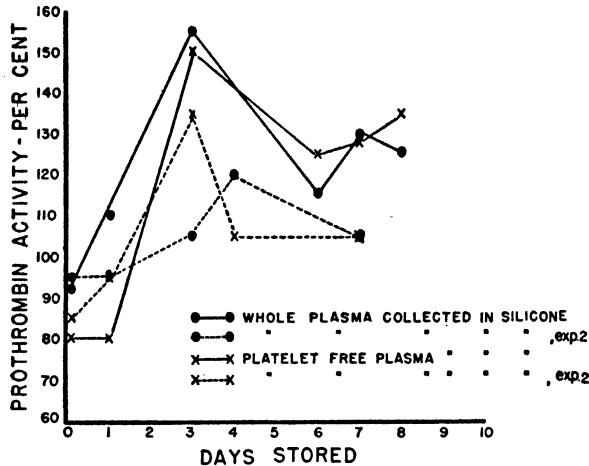


Fig. 4. PROTHROMBIC ACTIVITY, during storage at 4°-5° C., of platelet-rich and platelet-poor (centrifuged) oxalated human plasma.

elaboration of some accelerator the prothrombin time need not be altered. Accordingly, deterioration of labile factor and evolution of the accelerator during storage may be interrelated.

It is also noteworthy that prothrombin hyperreactivity consequent to aging cannot be demonstrated unless a component found in *fresh* plasma is simultaneously provided. This suggests that labile factor, or some other plasma substance similarly labile, is essential for the activity of the accelerator in speeding thrombin evolution. The same is true of the prothrombin conversion accelerator found in serum (7).

That the accelerator appears earlier in a prothrombin rich fraction than in the parent plasma suggests that its evolution may be retarded by the presence of one or more of the plasma proteins, or that the manipulation of fractionation may, somehow, render the precursor more susceptible to transformation.

⁷ Insufficiency of plasma Ac-globulin has been induced in dogs by injuring the liver with chloroform, resulting in an elevated prothrombin time (24).

Why hyperreactivity does not occur in plasma stored at room or body temperature in contrast to refrigerator temperature is obscure.

CONCLUSIONS

The prothrombic activity (one-stage) of normal plasma or prothrombin-rich fractions obtained by adsorption with BaSO_4 and elution with sodium citrate increases during storage at refrigerator temperature. This does not occur at room or body temperature. In some plasmas prothrombic hyperreactivity appears within 24 or 48 hours; in others it develops after two or more weeks of storage. In the latter instances, prothrombin-rich fractions become hyperreactive much earlier than the parent plasmas.

The phenomenon is attributable to evolution of an agent which accelerates the conversion of prothrombin to thrombin by thromboplastin plus calcium. The agent as well as its precursor(s) can, like prothrombin and the prothrombin conversion accelerator of serum, be adsorbed by BaSO_4 and eluted with sodium citrate. It arises in hemophilic plasma, thrombocytopenic plasma, and in normal plasma handled entirely in siliconized apparatus. The accelerator cannot be demonstrated in the absence of a labile factor present in fresh plasma.

Changes in stored dicumarolized plasma were not striking. Also, the accelerator did not evolve in plasma deprived of prothrombin by adsorption with BaSO_4 , or in hypoprothrombinemic plasma from a patient with severe hepatic cirrhosis. Its possible relationship to serum Ac-globulin, or the prothrombin conversion accelerator of serum, is discussed.

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THE IN VITRO RELEASE OF HISTAMINE FROM THE BLOOD CELLS OF SENSITIZED RABBITS: RELATIONSHIP TO BLOOD COAGULATION MECHANISMS¹

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HISTAMINE is released from the blood cells into the plasma when antigen is added *in vitro* to whole blood from the sensitized rabbit. This was first demonstrated by Katz (1) and has been considered by him and other workers (2) to be an *in vitro* anaphylactic type of reaction. It is well known also that histamine is released from the blood cells when rabbit blood is allowed to coagulate. In a study of the mechanism of the *in vitro* anaphylactic reaction, the possibility of participation by different components of the blood coagulation system must therefore be considered. In this paper are reported the results of our studies on the relationship between the anaphylactic histamine release mechanism and certain components of the blood coagulation system. Our evidence indicates that prothrombin, thrombin, thromboplastin and Ac-globulin are not involved in the anaphylactic histamine release. On the other hand, the mechanism of this reaction has some properties in common with the blood coagulation mechanism.

EXPERIMENTAL

Rabbits, 2 to 4 kg. in weight, were sensitized by the intramuscular injection of 0.2 ml. of antigen emulsion into each of 10 different sites on the same day. The antigen emulsion was of the Freund type (3) with constituents as follows: 7 mg. of dry heat-killed saprophytic acid-fast bacterial cells, 4 ml. heavy mineral oil, 2 ml. aquaphor, and 8 ml. of fresh egg white. In 10 to 14 days after the injection of antigen, the rabbits were exsanguinated through the carotid artery by means of a coated cannula, under local anesthesia. For each 50 ml. of blood collected 0.1 ml. of heparin solution (1000 units/ml.) was added. All glassware in which whole blood was handled was coated with either General Electric Dri-film 9987 or Dow-Corning Pan Glaze. The latter film is very durable and the glassware can be used repeatedly without being recoated.

Crystalline trypsin and crystalline soy trypsin inhibitor were kindly supplied by Dr. M. Kunitz. For some experiments the trypsin inhibitor preparations were made in our laboratory according to the procedure of Kunitz (4). The sodium salt of heparin, 100 units per milligram, kindly supplied by Dr. Ira B. Cushing of these laboratories, was used in all experiments. A 1 per cent solution of heparin was made up in saline *without a preservative*. The chloride salts of Ca^{++} and Mg^{++} were used as the sources of these ions.

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All *in vitro* histamine release reactions were carried out as follows. Constituents of the reaction in isotonic solution were pipetted into a heavy duty 12 ml. conical centrifuge tube and the volume was diluted to 2.6 ml. with saline. To this solution 2 ml. of whole blood was added, followed by thorough mixing, and then 1 ml. of a 1:100 dilution of whole egg white in saline was added. The contents of each tube were mixed well; the tubes were placed in a 37°C. water bath for 20 minutes and then in an ice bath. Blood cells were removed by centrifugation for 15 minutes in a refrigerated centrifuge. A 4.5 ml. aliquot of plasma was carried through the histamine purification procedure of McIntire, Roth and Shaw (5). At the end of the histamine purification, the cotton succinate eluates were not neutralized; the acid eluates were evaporated to dryness at reduced pressure and the histamine was determined by the chemical method of McIntire *et al.* (6). In our data the concentration of heparin,

TABLE 1. IN VITRO HISTAMINE RELEASE FROM BLOOD CELLS OF DICUMAROL-TREATED SENSITIZED RABBITS

RABBIT NO.	CLOTTING TIME	HISTAMINE RELEASED	
	minutes	μg/ml. of blood	% of total
920	10	1.79	72
921	> 25	1.75	54
922	20	1.44	41
923	12.5	1.4	29
924	24	1.2	42
926	18.5	3.21	68
Normals untreated	2 to 4	0.5 to 4.3	27 to 79

phenol, oxalate, citrate, Ca^{++} , Mg^{++} and trypsin inhibitor are given in terms of the final dilution in the *in vitro* reaction. The concentration per ml. of blood would be 2.5 times the concentration indicated.

RESULTS AND DISCUSSION

The *in vitro* histamine release by antigen can be carried out without any noticeable blood coagulation if silicone-coated glassware and a very small amount of heparin are used. However, this fact does not exclude the possibility that some of the enzymatic components of the coagulation mechanism might participate in the histamine release reaction. One of our first approaches to this question was the treatment of sensitized rabbits with high doses of dicumarol to decrease the prothrombin content of the blood as much as possible just short of fatal treatment.

The animals were given 10 mg. of dicumarol per kilo intravenously each day (usually for 5-6 days) until the blood-clotting time was greatly prolonged. They were then exsanguinated and the degree of anaphylactic *in vitro* histamine release was determined. The data of table 1 indicate that the heavy dicumarol treatment did not affect the anaphylactic histamine release mechanism. There is no relationship between the clotting time of the blood and the percentage of the total histamine released by antigen. The percentage of total histamine released from blood of both dicumarol-treated rabbits and untreated rabbits is essentially the same. Since it is well known that dicumarol treatment sufficient to prolong markedly the blood-clotting time

greatly decreases the prothrombin content of the blood, we may conclude that a great decrease in the prothrombin content does not impair the anaphylactic histamine release mechanism.

The inhibition of the *in vitro* anaphylactic reaction by heparin was first reported by Dragstedt *et al.* (2). The amount of heparin they used to inhibit the reaction (0.12%) was much higher than the amount required to prevent coagulation. We have determined the inhibition of histamine release by various levels of heparin and the data are shown in figure 1. The heparin concentrations indicated represent the concentration over and above the 0.008 mg/ml. which was used in all experiments

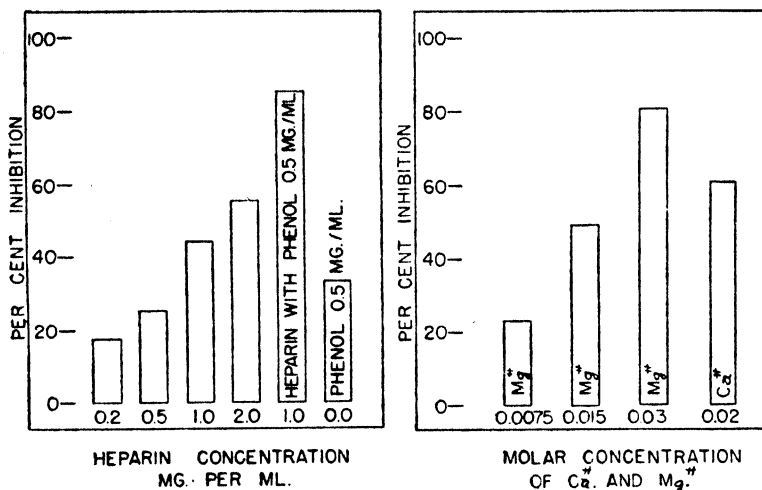


Fig. 1 (left). HEPARIN inhibition of the *in vitro* 'anaphylactic' histamine release.

Fig. 2 (right). INHIBITORY effect of Mg^{++} and Ca^{++} on the *in vitro* release of histamine by antigen.

to prevent blood clotting. These data are an average of several experiments. The deviation from the average values is not great when the histamine release in the controls is average or above. In experiments with very poor histamine release the inhibition by the lower levels of heparin is greater percentage-wise. These data indicate that the amount of heparin necessary to give a 55 per cent inhibition of the anaphylactic histamine release is at least 250 times the amount required to prevent blood clotting for several hours under these conditions, and approximately 1000 times the amount of heparin required to inhibit markedly the action of thrombin and thromboplastin in more purified systems (7). The weak inhibition of the histamine release reaction by heparin very strongly suggests that neither thrombin nor thromboplastin is important in the *in vitro* anaphylactic reaction. The data on the inhibition by *phenol* and *heparin + phenol* are included to point out the potential error had we used a commercial heparin containing phenol as a preservative. One mg/ml. of heparin with the usual amount of phenol used as a preservative gives a much greater inhibition than 2 mg/ml. of heparin without phenol. In fact, milligram for milligram the phenol is a more potent inhibitor than is heparin.

Further evidence against the participation of thromboplastin and Ac-globulin

in the histamine release reaction is the fact that the soy bean trypsin inhibitor fails to inhibit this reaction (table 2). MacFarlane (7) found that a concentration of 0.01 mg/ml. of soy trypsin inhibitor markedly inhibited the conversion of prothrombin to thrombin. Our data show that even 1.6 mg/ml. of the trypsin inhibitor does not significantly inhibit histamine release by antigen, while only 0.4 mg. of inhibitor per ml. almost completely inhibits histamine release by an optimal concentration of trypsin. Since the soybean trypsin inhibitor is a potent inhibitor for both Ac-globulin and thromboplastin (8) these components of the blood coagulation system very likely do not participate in the *in vitro* anaphylactic histamine release.

The failure of soy trypsin inhibitor to inhibit the histamine release by antigen is also concrete evidence against the popular theory that the histamine release depends

TABLE 2. EFFECT OF SOYBEAN TRYPSIN INHIBITOR ON HISTAMINE RELEASE BY ANTIGEN AND BY TRYPSIN

HISTAMINE RELEASE AGENT	SOY TRYPSIN INHIBITOR mg/ml.	% INHIBITION
Antigen	0.8	7
	1.6	7
Trypsin		
0.08 mg/ml.	0.4	95
0.15 mg/ml.	0.4	74

TABLE 3. OXALATE AND CITRATE INHIBITION OF HISTAMINE RELEASE BY ANTIGEN

INHIBITOR	MOLAR CONCENTRATION	% INHIBITION	INHIBITOR	MOLAR CONCENTRATION	% INHIBITION
Oxalate	0.002	22	Citrate	0.0017	42
	0.006	100		0.0051	96
	0.01	100		0.0068	100
				0.0085	100

upon the activation of the plasma protease, fibrinolysin. This point will be considered more completely in a later publication.

The histamine release mechanism has some properties in common with the blood coagulation system in being inhibited by oxalate, citrate, Mg^{++} and Ca^{++} as shown in figure 2 and table 3. The concentration of Mg^{++} which gives an 80 per cent inhibition of histamine release will infinitely prolong the clotting time of rabbit plasma (9), and the amount of Ca^{++} which will prolong the clotting time of rabbit blood to more than 60 minutes (9) gives a 60% inhibition of the histamine release. The concentrations of oxalate and citrate which are required for a 100 per cent inhibition of the histamine release reaction are of the same order as are required for effective anti-coagulant action.

There is, however, one important difference in the effect of citrate on the two mechanisms under consideration. While there is a slow inactivation of some component of the blood coagulation system under the influence of 0.02M citrate (10), there is a very rapid inactivation of the histamine release mechanism in a much lower

concentration of citrate (0.0068M). The data of table 4 show that when calcium chloride is added to citrated blood in only one minute after the citrate has been added, the inhibition by citrate is nearly 60 per cent irreversible. If the citrate is allowed to react with the blood for 20 minutes before the addition of Ca^{++} , the inhibition is 75 per cent irreversible. Attempts to reverse the oxalate inhibition resulted in coagulation, hemolysis and the release of histamine before antigen was added.

The fact that oxalate, citrate, Ca^{++} and Mg^{++} inhibit the anaphylactic release of histamine does not necessarily mean that this reaction involves any part of the blood coagulation system. The rapid irreversible citrate inhibition of the anaphylactic

TABLE 4. IRREVERSIBILITY OF CITRATE INHIBITION OF HISTAMINE RELEASE BY ANTIGEN

MOLAR CONCENTRATION	PERCENTAGE INHIBITION		MOLAR CONCENTRATION	PERCENTAGE INHIBITION	
	1 min. ¹	20 min. ¹		1 min. ¹	20 min. ¹
Citrate 0.0068	97	100	Citrate 0.0068		
			CaCl_2 0.006	57	69
Citrate 0.0068			Citrate 0.0068		
CaCl_2 0.004	56	79	CaCl_2 0.008	74	76

¹ This indicates the length of time citrate was allowed to react with the blood before Ca^{++} was added.

histamine release indicates that the histamine release mechanism differs from the blood coagulation system with respect to the components affected by citrate.

SUMMARY

Thrombin, prothrombin, thromboplastin and Ac-globulin probably are not involved in the *in vitro* release of histamine by antigen from the blood cells of sensitized rabbits because: a) severe dicumarol treatment does not affect the histamine release, b) extremely large amounts of heparin only partially inhibit the histamine release, and c) soy bean trypsin inhibitor fails to inhibit the histamine release.

Oxalate, citrate, Ca^{++} and Mg^{++} inhibit the histamine release reaction at approximately the same concentrations as are required for effective anticoagulant action. The histamine release mechanism is much more rapidly inactivated by citrate than is any part of the blood coagulation system.

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COLORIMETRIC DETERMINATION OF LIPASE AND ESTERASE IN DOG'S SERUM¹

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THE evidence has become increasingly convincing that there are two distinct esterolytic enzymes capable of splitting a wide variety of carboxylic acid esters (1-4). One enzyme, esterase, is abundant in liver, kidney, blood serum and pancreas, hydrolyzes esters of short chain fatty acids with greater facility than esters of long chain fatty acids and is inhibited by fluoride (5), atoxyl (6, 7), and taurocholate (8, 9). The other enzyme, lipase, is abundant almost exclusively in pancreas, hydrolyzes esters of long chain fatty acids (C₈-C₁₈), (2, 4) is inhibited by quinine (10) and specifically accelerated by taurocholate (4, 8). Fatty substances such as tributyrin are hydrolyzed by both enzymes (11). Even olive oil, which is considered to be an ideal substrate for lipase, is hydrolyzed to a slight extent by esterase.

These facts account for the lack of specificity possessed by current methods for measuring lipase, which utilize tributyrin (12), 'Tween' (13) or more reliably, olive oil, as substrates. The fatty acid produced by enzymatic hydrolysis is determined by titration with N/20 sodium hydroxide (14). This is not an easy or convenient procedure to perform accurately, especially in the presence of serum protein and a heavy emulsion. A reliable method for measuring serum lipase would provide a valuable tool for the study of pancreatic disease.

A recent study of esterase and lipase activity of the tissues of several species by the use of three chromogenic substrates suggested the possibility of developing a convenient, sensitive and specific method for measuring serum lipase and esterase (4). It has been shown that two injections of mecholyl and eserine at 15-minute intervals produced a regular increase in the hydrolytic activity of dog serum upon olive oil (15). This technique has been modified by the use of acetic (C₂), lauric (C₁₂), and palmitic-stearic (C₁₆-C₁₈) acid esters of beta naphthol, instead of olive oil, as substrates (4). Following enzymatic hydrolysis, beta naphthol is coupled with tetrazotized diorthoanisidine to form a purple azo dye, which is extracted from the aqueous medium with ethyl acetate and measured in a photoelectric colorimeter (4). This method for serum lipase and esterase has been studied in dog's serum and forms the basis of this report.

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² Research fellow of the National Cancer Institute.

METHOD

Venous blood (10 cc.) was taken from 11 mongrel dogs. Immediately following this, two subcutaneous injections at 15-minute intervals were made of acetyl beta methylcholine hydrochloride, and of eserine sulfate (0.05 mg/kg. body weight each). Blood samples were taken after 1 hour and again after 2 hours. In two experiments specimens were also taken 7 and 24 hours later. The clotted blood was centrifuged at 2000 rpm for 15 minutes, and the serum was diluted with water for the determination of esterase and lipase (table 1).

The synthesis of the substrates, beta naphthyl acetate, beta naphthyl laurate, and beta naphthyl palmitate-stearate, is given elsewhere (4, 16). Each substrate (10 mg.) was dissolved separately in acetone (2 cc. for the acetate or 10 cc. for the laurate or palmitate-stearate) and introduced through a submerged pipette into a swirling mixture of 20 cc. veronal buffer³, pH 7.4 and water (final volume 100 cc.). The final concentration of substrate was 0.1 mg/cc. To 5 cc. of each of these solutions a quantity of diluted serum (see table 1) was added. Incubation was then conducted for the time and at the temperature given in table 1. Different concentra-

TABLE 1

SUBSTRATE	SERUM DILUTION FACTOR	PERIOD OF INCUBATION	TEMPERATURE OF INCUBATION
		hr.	°C.
Beta naphthyl acetate.....	40	$\frac{1}{2}$	23-27
Beta naphthyl laurate.....	20	2	37.5
Beta naphthyl palmitate-stearate.....	10	24	37.5

tions and conditions for each substrate were required because of the widely differing rates of enzymatic hydrolysis of these esters. At the end of the period of incubation, 1 cc. (4 mg.) of a freshly prepared, cool solution of tetrazotized diorthoanisidine⁴ was added and shaken into each tube. A purple azo dye formed immediately. Forty per cent trichloroacetic acid (1 cc.) was then added to break the protein-azo dye complex, and the pigment was extracted by shaking with 10 cc. ethyl acetate. The tubes were centrifuged for 15 minutes at 1500 rpm and 5 cc. of the clear, purplish-red organic layer was transferred to a colorimeter tube with a pipette, and measured with a photoelectric colorimeter (Klett) through a 540 m μ filter. From a calibration curve of pure beta naphthol, which is linear between 0.005 to 0.01 mg., color density was converted to milligrams and micromoles of naphthol.

In experiments in which inhibitors of the reaction or activators were studied, the diluted serum was incubated with 1 cc. of the appropriate agent for 30 minutes at room temperature before the substrate solution was added. These agents were

³ Prepared (17) by mixing 66.5 cc. of a solution containing 10.3 gm. of sodium diethyl barbiturate in 500 cc. of distilled water with 33.5 cc. of an 0.1 M hydrochloric acid solution.

⁴ Available commercially in powder form, containing 20% tetrazotized diorthoanisidine, 5% zinc chloride and 20% aluminum sulfate, under the trade name, Dupont Naphthanil Diazo Blue B. Provided through the courtesy of Dr. E. R. Laughlin, Dupont de Nemours and Co., Boston, Mass.

stored in the refrigerator in the following concentrations: sodium fluoride, 30 mg/cc.; sodium taurocholate, 2.2. mg/cc.; and quinine sulfate, 40 mg/cc.

RESULTS

The sera of 11 dogs showed little change in ability to hydrolyze beta naphthyl acetate after injection of mecholyl and eserine. However, with naphthyl laurate and naphthyl palmitate-stearate, the sera of 7 dogs showed an appreciable increase in esterolytic activity after mecholyl and eserine, 2 showed a slight increase in enzymatic activity and 2 were unaffected. The data in 4 experiments which showed a significant elevation in enzymatic activity are given in table 2. These increases were more striking when the sera were exposed to sodium taurocholate prior to

TABLE 2. ESTEROLYTIC ACTIVITY OF DOG SERUM BEFORE AND AFTER INJECTION OF MECHOLYL AND ESERINE

SUBSTRATE	EXPER. NO.	MICROMOLES OF NAPHTHOL LIBERATED PER CC. OF SERUM/HOUR		
		Before Injection	1 Hour After Injection	2 Hours After Injection
Beta naphthyl acetate.....	3	39	41	41
	4	24	27	31
	9	75	92	92
	10	55	58	66
Beta naphthyl laurate.....	3	0.43	0.63	0.49
	4	0.28	0.42	0.42
	9	0.76	2.7	2.4
	10	0.49	0.77	0.63
Beta naphthyl palmitate-stearate.....	3	0.03	0.04	0.03
	4	0.00	0.01	0.01
	9	0.06	0.22	0.14
	10	0.03	0.10	0.05

incubation with the substrates (table 3). Under these conditions, the sera of 9 of 11 dogs showed marked increases in esterolytic power for all 3 esters after mecholyl and eserine injection (fig. 1). The rises in esterolytic power caused by sodium taurocholate were 12 to 228 per cent (acetate), 33 to 620 per cent (laurate), and 42 to 900 per cent (palmitate-stearate).

In 2 dogs followed for 24 hours after mecholyl and eserine injection (fig. 1), a slow fall in esterase and lipase activity was noted between 2 and 6 hours. Normal levels were reached within 24 hours.

Further evidence for the specificity of action of the enzyme which appeared in increased amount in the serum after mecholyl and eserine was provided by experi-

* Liver and pancreas were homogenized and diluted with water so that each cc. contained the following wet weight (1) of liver: 0.25-0.5 mg. for acetate substrate, 1.0 mg. for laurate, and 5.0 mg. for palmitate-stearate; and (2) of pancreas: 0.1-0.2 mg. for acetate and laurate, and 0.1 mg. for the palmitate-stearate.

ments with taurocholate, fluoride and quinine. Homogenates⁵ of liver and pancreas were prepared and tested in exactly the same way as serum (table 4).

The esterolytic action of liver was inhibited by sodium taurocholate, whereas that of pancreas and serum (S₁) was accelerated. Serum after injection of mecholyl

TABLE 3. ESTEROLYTIC ACTIVITY OF DOG SERUM ACTIVATED BY SODIUM TAUROCHOLATE BEFORE AND AFTER INJECTION OF MECHOLYL AND ESERINE

SUBSTRATE	EXPER. NO.	MICROMOLES OF NAPHTHOL LIBERATED PER CC. OF SERUM/HOUR		
		Before Injection	1 Hour After Injection	2 Hours After Injection
Beta naphthyl acetate	3	55	72	80
	4	30	85	98
	9	73	117	117
	10	68	117	117
Beta naphthyl laurate	3	3.5	6.6	8.5
	4	1.2	9.0	9.8
	9	7.6	9.7	9.8
	10	3.5	9.7	9.8
Beta naphthyl palmitate-stearate	3	0.08	0.27	0.35
	4	0.04	0.38	0.41
	9	0.15	0.41	0.41
	10	0.04	0.41	0.41

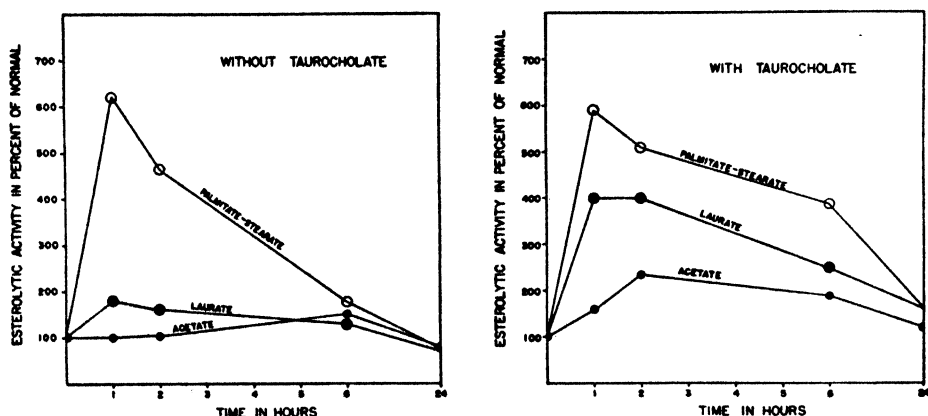


Fig. 1. THE FIRST GRAPH shows the esterolytic activity of dog serum on 3 substrates after the injection of mecholyl and eserine. The data is plotted in percentage of the activity of the pre-injection level and shows that the enzyme which appears in the serum hydrolyzes preferentially the higher fatty acid esters. The second graph shows the stimulating action of taurocholate on the hydrolytic activity of the enzyme (lipase) which appears in the serum after injection of mecholyl and eserine. The peaks of the rises were actually higher than shown (beyond range of the colorimeter). The greatest effect of taurocholate is shown with beta naphthyl laurate.

and eserine (S₂) was accelerated even more, suggesting that the increased hydrolytic activity of serum was due to an enzyme (lipase) found in pancreas and not in liver.

Sodium fluoride inhibited markedly the action of the tissues and serum on the 3 substrates. However, when both taurocholate and fluoride were added, although inhibition of hydrolysis was still observed in liver and serum (S_1), accentuation was observed with pancreas on all 3 substrates, and with serum (S_2) on the laurate and palmitate-stearate esters. Thus the activating effect of taurocholate on lipase was apparent even in the presence of fluoride.

Quinine inhibited esterolysis, except for two instances of acceleration; i.e. pancreas on the acetate substrate, and liver on the palmitate-stearate substrate. With both taurocholate and quinine, the quinine effect was the same but more pronounced except for one instance of reversal; i.e. increased hydrolysis of naphthyl laurate by pancreas.

TABLE 4. EFFECTS OF CERTAIN AGENTS UPON ESTEROLYTIC ACTIVITY OF LIVER, PANCREAS, AND SERUM IN THE DOG¹

REAGENTS	BETA NAPHTHYL ACETATE				BETA NAPHTHYL LAURATE				BETA NAPHTHYL PALMITATE-STEARATE			
	Liver	Pan- creas	Serum 1 ²	Serum 2 ²	Liver	Pan- creas	Serum 1 ²	Serum 2 ²	Liver	Pan- creas	Serum 1 ²	Serum 2 ²
Sodium tauro- cholate	oo	++	+	++	o	++	++	+++	o	+	+	++++
Sodium fluoride	ooo	ooo	ooo	ooo	ooo	ooo	ooo	ooo	oo	oo	ooo	ooo
Sodium fluoride and sodium taurocholate	oo	+	oo	oo	oooo	++	o	++	o	++	o	++
Quinine	o	+	ooo	ooo	o	oooo	ooo	ooo	+++	oooo	ooo	ooo
Quinine and so- dium tauro- cholate	ooo	++	oooo	ooo	oo	+	ooo	ooo	+++	oooo	oooo	oooo

¹ Symbols have the following meaning:

Inhibition Acceleration

o + slight
oo ++ strong
ooo +++ marked
oooo ++++ maximum

² Serum 1 refers to serum taken before injection of eserine and mecholyl, while serum 2 refers to serum taken 1 hour following injection.

DISCUSSION

The work of several investigators (1-4) indicated that the esterolytic activity of liver and serum differed from that of pancreas according to the type of substrate which was readily hydrolyzed by each. Other evidence for the specificity of pancreatic lipase was afforded by the experiments of Cherry and Crandall (14), and later by Nothman, Pratt and Benotti (18), in which an increase in the amount of enzyme able to split olive oil was demonstrated in the serum of the dog after pancreatic injury, whereas no increase in ethyl butyrase activity was demonstrated under these conditions. Similar changes were reported by Popper and Necheles (15) after injection of mecholyl and eserine. The experiments with the chromogenic substrates reported here confirm these observations.

Since esterase may hydrolyze olive oil to a slight extent and lipase may hydrolyze esters of short chain fatty acids, more conclusive proof of the specificity of the lipase activity in serum was needed. This was provided by the experiments with taurocholate and to a lesser degree with the other accelerators or inhibitors. Since taurocholate inhibits the esterolytic action of liver (esterase) and accelerates the activity of pancreas (lipase), acceleration of the esterolytic activity of serum, particularly after mecholyl and eserine injection, indicates that the enzyme appearing in the blood is pancreatic in origin (lipase). The taurocholate effect not only adds specificity to the serum lipase determination but increased sensitivity as well.

The strong hydrolytic action of serum on naphthyl acetate demonstrates esterase; the smaller hydrolytic action of serum on naphthyl laurate and palmitate-stearate demonstrates both esterase and lipase. Liver acts similarly. However, the constant accelerating effect of taurocholate upon serum and its inhibitory effect on liver indicates that lipase is present in normal serum but not in liver, within the limits of this method.

While human liver and pancreas act the same as dog liver and pancreas, human serum differs from dog serum in that lipase is not demonstrable by this technique in normal serum. The determination of serum lipase and esterase in man will form the subject of another communication (19).

SUMMARY

Methods for the colorimetric determination of esterase and lipase in the serum of dogs are given. Increase in lipase content of serum was demonstrated in dogs after injection of mecholyl and eserine. Evidence that esterase and lipase can be separately determined in serum was provided by experiments with specific accelerators and inhibitors of enzymatic activity in serum, liver, and pancreas.

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LIVER REGENERATION IN THE PRESENCE OF COMMON BILE DUCT OBSTRUCTION¹

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HIGGINS and Anderson (1) working with rats and Mann, Fishback, Gay and Green (2), with dogs concluded that little or no parenchymal liver regeneration occurred when biliary flow was obstructed. As a result of the following experiments, we believe that liver regeneration does occur in the rat in the early mid-stages of biliary stasis to a degree equal to that occurring in pair-fed unobstructed controls. This is based on observations of liver mass (both wet and dry), liver protein and mitotic activity.

METHODS

Male Wistar rats, averaging 250 grams in body weight, were placed on a synthetic non-protein diet (G-2) for 14 days. The animals, under ether anesthesia, were subjected to a 70 per cent partial hepatectomy, followed immediately by high ligation and division of the common bile duct. Animals partially hepatectomized only served as controls.

Postoperatively, four groups of biliary obstructed rats were fed a 10 per cent casein, low fat diet (G-6) while control groups were fed the same diet, some *ad libitum*, and others limited to the amount eaten by the obstructed animals. The rats were killed on the 2nd, 4th, 8th and 14th postoperative days under intra-peritoneal sodium amytal anesthesia. At both operation and autopsy, liver mass (both in the wet and dry state) was determined, and analyses for protein, glycogen and lipid were carried out as previously described (3). At autopsy, blood was obtained from the inferior vena cava for serum protein, prothrombin and icterus index determinations. All livers were examined histologically. Other groups of rats subjected to the same preoperative and operative procedures as those described were fed postoperatively, 1) a non-protein, low fat diet (G-2); 2) a high protein, low fat diet (G-1); and 3) a high fat (30%), 13.2 per cent protein diet (F-4) with and without supplemental desiccated pig bile⁴ (0.5%). These rats were killed on the 14th postoperative day. The composition of all diets is given in table 1.

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⁴ Desicol (Parke, Davis & Co.).

One group of rats after 14 days of protein depletion was subjected to high ligation and division of the common bile duct alone, without partial hepatectomy. They were fed diet G-6 and were killed on the 14th postoperative day. Control rats, pair-fed to these, had laparotomy only.

One further group of rats after 7 days' protein depletion had ligation of the common bile duct and after a second 7-day period of protein depletion were subjected to partial hepatectomy.⁵ They were then fed a 10 per cent casein low fat diet (G-6) and were killed on the 14th day following the second operation. Parenteral vitamin K (Synkovite 1 mg.) was administered to each of these rats on three occasions near the time of their second operation.

Food consumption and body weight of all rats were recorded daily. Four days prior to operation and during the entire postoperative period, nitrogen analyses of feces and urine were done daily (4).

TABLE 1

	G-2	G-6	G-1	F-4
	%	%	%	%
Sucrose.....	91	81	73	48.9
Casein.....	0	10	18	13.2
Cod liver oil.....	3	3	3	3
Salts.....	4	4	4	5.3
Cellulflour.....	2	2	2	2.6
Mazola.....				2
Crisco.....				25
Cal/gm.....	3.9	3.9	3.9	5.2

Composition of Diets. All diets contained a vitamin supplement consisting of the following quantities per 10.0 gm. unit of food: thiamine, riboflavin and pyridoxine, 0.1 mg. each; nicotinic acid, 1.0 mg.; inositol, 6.0 mg.; para-aminobenzoic acid 2.0 mg.; calcium pantethenate 0.6 mg.; and choline chloride 20.0 mg. In diet F-4, the amount of choline was doubled. All biliary obstructed animals received also oral vitamin K (Menadione) 0.05 mg/10 gm. of food.

RESULTS

Essential data are summarized in table 2. Comparison of the 10 per cent casein-fed (G-6) biliary obstructed and control rats, killed on the 2nd, 4th, 8th and 14th postoperative days (*exper. 1*), is facilitated by the diagrams shown as figures 1 and 2.

Figure 1 shows the amounts of liver protein regeneration. All biliary obstructed animals suffered approximately a 50 per cent reduction of appetite, and, accordingly, some controls were pair-fed (limited) with the jaundiced rats, and others were fed *ad libitum*. It is seen throughout all postoperative periods that the biliary obstructed rats regenerated more liver protein than did unobstructed rats, whether pair-fed, or fed *ad libitum*.

A portion, however, of the protein regenerated in obstructed rats was due to

⁵ From other studies, the median and left lateral lobes comprised 65 per cent (not 70%) of the total liver mass when biliary outflow was obstructed for this period.

TABLE 2. DATA OF BILIARY OBSTRUCTED AND UNOBSTRUCTED CONTROL RATS

EXPER. NO.	POSTOP. PERIOD	DIET	OPERATION	NO. OF RATS	INITIAL BODY WT.	POSTOP. WT. GAIN OR LOSS	LIVER REGENERATION ¹			NITRO-GEN ¹		PROTEIN	SE- RUM Prothrombin	ICTERUS INDEX	MITOSES/1000 LIVER NUCLEI
							Wet mass	Dry mass	Protein	Intake	Balance				
					gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm. %	%		
1A	2	G-6 10% Casein	Part-hep. C.B.D. ligat.	6	251 ±11	-9 ±3	0.71 ±.18	0.14 ±.01	0.13 ±.01	0.04 ±.01	-0.03 ±.02	4.66	80		10.3 ±2.1
	2 ^a	Non-protein	Part-hep.	5	242 ±11	-8 ±2	0.68 ±.10	0.19 ±.01	0.10 ±.01	0 0		4.43			10.9 ±4.0
	4	G-6 10% Casein	Part-hep. C.B.D. ligat.	6	255 ±13	-9 ±7	1.20 ±.13	0.26 ±.01	0.23 ±.01	0.09 ±.01	-0.04 ±.01	5.01	84		11.7 ±3.0
1B	4	G-6 Limited 10% Casein	Part-hep.	5	247 ±7	-9 ±2	0.65 ±.07	0.14 ±.02	0.20 ±.01	0.00 ±.01	-0.04 ±.01	4.46	100		1.0 ±1.2
	4 ^a	Non-Protein	Part-hep.	5	247 ±11	-7 ±4	0.90 ±.16	0.27 ±.10	0.14 ±.01	0 0		4.64			
	8	G-6 10% Casein	Part-hep. C.B.D. ligat.	5	251 ±10	-7 ±6	1.86 ±.42	0.39 ±.14	0.32 ±.04	0.24 ±.04	-0.06 ±.08	5.33	98	36	2.8 ±1.4
1C	8	G-6 Limited 10% Casein	Part-hep.	6	247 ±11	-15 ±2	0.78 ±.14	0.21 ±.03	0.23 ±.02	0.24 ±.01	-0.05 ±.02	5.38	95		0.2
	8	G-6 <i>Ad lib.</i>	Part-hep.	6	272 ±6	+7 ±5	1.46 ±.17	0.39 ±.07	0.26 ±.01	0.40 ±.04	+1.10 ±.03	5.29	93		0.6
	8	10% Casein	Part-hep.	6	250 ±24	-7 ±8	2.12 ±.23	0.45 ±.07	0.36 ±.04	0.46 ±.08	-0.02 ±.06	5.78	82	51	2.5 ±1.9
1D	14	G-6 Limited 10% Casein	Part-hep.	5	261 ±15	-12 ±3	0.79 ±.08	0.22 ±.07	0.25 ±.02	0.46 ±.03	0.01 ±.03	5.53	100	19	0.4
	14	G-6 <i>Ad lib.</i>	Part-hep.	5	245 ±10	+34 ±9	2.01 ±.16	0.60 ±.05	0.34 ±.03	0.98 ±.13	0.27 ±.05	5.19			
	14	10% Casein	Part-hep.	5	250 ±24	-7 ±8	2.12 ±.23	0.45 ±.07	0.36 ±.04	0.46 ±.08	-0.02 ±.06	5.78	82	51	2.5 ±1.9
2	14	G-2 Non-Protein	Part-hep. C.B.D. ligat.	8	262 ±18	-27 ±11	1.50 ±.22	0.30 ±.03	0.23 ±.02	0 0	-0.33 ±.06	4.88	70	36	
	14	G-1	Part-hep.	8	246 ±17	+17 ±15	2.87 ±.44	0.63 ±.12	0.52 ±.08	1.09 ±.24	+0.33 ±.16	5.07	93	49	
	14	18% Casein	C.B.D. ligat.	9	246 ±21	+17 ±15	2.87 ±.44	0.63 ±.12	0.52 ±.08	1.09 ±.24	+0.33 ±.16	5.07	93	49	
3	14	F-4 30% Fat	Part-hep. C.B.D. ligat.	4	260 ±10	30 ±11	2.10 ±.29	0.43 ±.06	0.37 ±.03	0.32 ±.07	-0.26 ±.04	5.58	70	51	
	14	F-4 + Bile	Part-hep.	4	253 ±11	-33 ±11	2.14 ±.25	0.41 ±.05	0.36 ±.03	0.34 ±.12	-0.31 ±.08	5.70	76	50	
	14	30% Fat	C.B.D. ligat.	4	253 ±11	-33 ±11	2.14 ±.25	0.41 ±.05	0.36 ±.03	0.34 ±.12	-0.31 ±.08	5.70	76	50	
4	14	G-6 10% Casein	C.B.D. ligat. 1 week Part-hep.	3	255 ±15	+17 ±17	1.99 ±.43	0.50 ±.10	0.31 ±.03	0.68 ±.16	+1.18 ±.13	5.61	73	35	
	14	G-6 10% Casein	C.B.D. ligat.	6	253 ±6	+6 ±6	4.71 ±.39	1.06 ±.08	0.71 ±.05	0.64 ±.11	+0.09 ±.09	6.46	76	62	
	14	G-6 Limited 10% Casein	Laparotomy	5	253 ±9	+5 ±4	2.32 ±.09	0.68 ±.03	0.50 ±.02	0.62 ±.02	+1.10 ±.03	5.97	94		

¹ Liver regeneration and nitrogen intake and balances are expressed in gm/100 gm. initial body weight. Values shown are averages for each experimental group. Where individual values were determined, the standard deviation is shown: S.D. = $\sqrt{\frac{\sum (x - \bar{x})^2}{n-1}}$

^a Data previously published as *experiment 2A* and *3B* respectively, by Gurd, Vars and Ravdin (3).

bile duct proliferation and fibrosis, which was always observed. Some, too, might be associated with the increased fluid content of these livers either as plasma or lymph. In figure 1, the shaded non-outlined segment of the bars represents an estimation of

this non-parenchymal protein. It is seen that even with the subtraction of this portion, liver protein regeneration in the biliary obstructed livers occurs apace of the pair-fed controls but lags behind those fed *ad libitum*. No accurate method of calculating this portion of protein was found. The estimation of the extra fibrous tissue and duct

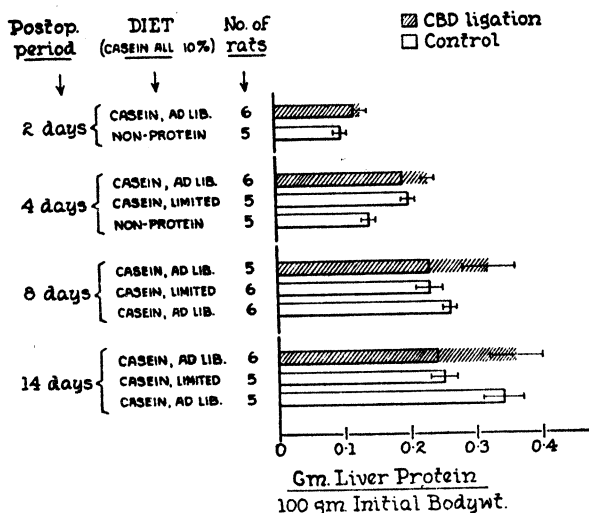


Fig. 1. LIVER PROTEIN REGENERATION in biliary obstructed and control rats following partial hepatectomy.

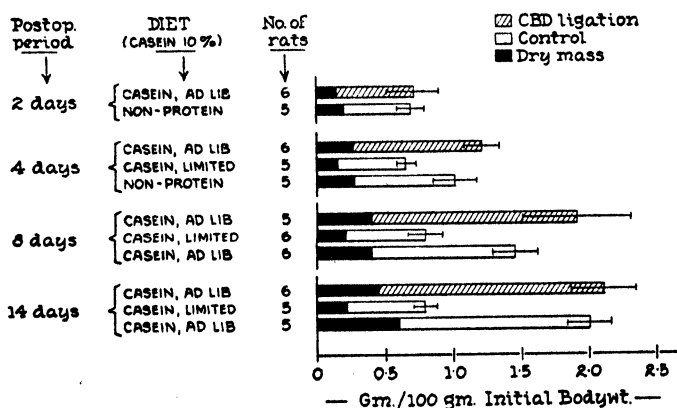


Fig. 2. LIVER MASS REGENERATION in biliary obstructed and control rats following partial hepatectomy.

protein was made from the histological appearance of the livers (Masson's trichrome, and Laidlaw's reticulum stain), while the extra fluid present in the biliary obstructed livers was considered to contain at most 8 per cent protein.

All biliary obstructed rats and pair-fed controls were in negative total nitrogen balance to an equal degree, while *ad libitum*-fed controls maintained a positive balance. Jaundice per se did not significantly increase the degree of negative nitrogen

balance over pair-fed non-jaundiced controls, when both had been previously protein depleted. This was not the case, however, when both groups had been well fed previously.

Figure 2 shows the amounts of liver mass regeneration (both wet and dry) occurring in these groups of rats. It is evident that, by weight as well as by protein, the biliary obstructed rats regenerated more than did both pair-fed and *ad libitum* fed controls.

Mitotic cell counts were made on sections of the livers from these groups of rats by the method used by Brues and Marble (5), that is the number of mitotic nuclei per 1000 hepatic cell nuclei. These counts were made under oil immersion, and approximately 20 fields dispersed throughout all parts of each section were required to be examined in order to count 1000 liver cells. Duct-cell mitoses were distinguished and excluded. Figure 3 graphically demonstrates mitotic activity in both biliary obstructed and unobstructed livers from groups of rats fed comparable diets. Mitotic

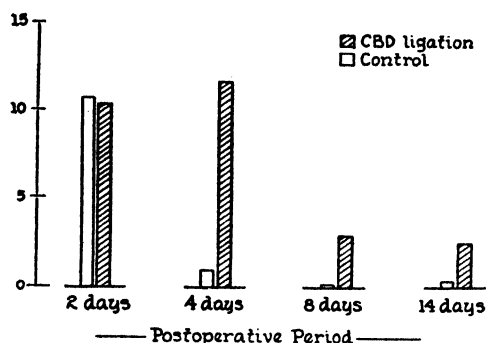


Fig. 3. NUMBER OF MITOSES PER 1000 HEPATIC CELL NUCLEI in biliary obstructed and control rats following partial hepatectomy.

counts of parenchymal cells in biliary obstructed livers and unobstructed controls were equal at the 2nd postoperative day. From that point on, mitotic activity in the controls rapidly decreased until mitoses were rare on the 8th and 14th postoperative days. In the obstructed livers, however, mitotic activity reached a peak on the 4th postoperative day and from then on decreased, but remained much higher than did that of the controls. It may be that the continued insult to the obstructed livers of the jaundice, and the increasing biliary distention, stimulated the parenchymal cells to greater regenerative efforts.

The biliary obstructed rats, which were fed various diets and killed 14 days postoperatively (*exper. 2*) regenerated liver protein in proportion to the amount of protein in the diet. With non-protein (G-2), 10 per cent casein (G-6), and 18 per cent casein (G-1) diets, liver protein regeneration was 0.23, 0.36 and 0.52 gm/100 gm. of initial body weight respectively. Vars and Gurd (3, 4, 6) obtained similar but lower results in unobstructed rats.

Two groups of biliary obstructed and partially hepatectomized rats (*exper. 3*) were fed a high fat diet containing 13.2 per cent casein (F-4). One of these groups received desiccated pig bile (Desicol) 0.5 per cent in the diet. When killed, 14 days

postoperative, no significant difference was noted in these two groups either in liver mass, liver protein, dietary intake or nitrogen balance. They regenerated liver protein (0.36 gm/100 gm. of initial body weight) equal to that regenerated by rats fed a low fat diet, although their nitrogen and caloric intake was less.⁶ They were, however, in marked negative nitrogen balance and in poor physical condition. Total postoperative fecal fat determinations were made on the two groups fed a high fat diet.⁷ A slight but greater fecal fat excretion occurred in the group fed bile, although the fat intake of the two groups was practically equal. Fecal nitrogen excretion was unaffected. It may be that the presence of bile in the diet stimulated intestinal motility, so that there was less time for fat absorption. Fecal fat excretion in biliary obstructed rats fed a 3 per cent fat diet compared to unobstructed pair-fed controls was found to be twice as great.⁸

One group of rats, having been protein-depleted for 14 days, then had their common bile ducts ligated, but without partial hepatectomy (*exper. 5*). They were then re-alimented on a 10 per cent casein low fat diet (G-6). A control group, subjected to laparotomy alone, was pair-fed with the above. When killed, on the 14th postoperative day, the biliary obstructed rats had livers which weighed wet 4.71 ± 0.39 gm., dry 1.06 ± 0.08 gm., and contained 0.71 ± 0.05 gm. of protein, all per 100 gm. of initial body weight. Control livers weighed wet 2.32 ± 0.09 gm., dry 0.68 ± 0.03 gm., and contained 0.50 ± 0.02 gm. of protein. The nitrogen intake and balance in both were identical. These two groups of rats were subjected to similar procedures in all respects, except for the addition of common bile duct ligation in one. Upon the assumption that their liver protein restitution should have been the same, the difference in liver protein between the biliary obstructed livers (0.71 gm.) and the unobstructed controls (0.50 gm.) may be considered to be the protein of duct-proliferation, fibrosis and edema. The factor .704 corrects for this portion of protein.

Using this factor to correct all 14-day common bile duct ligated and partially hepatectomized rats fed various diets, we may presume to obtain relatively true protein regeneration values of 0.16, 0.25, and 0.37 grams with non-protein (G-2), 10 per cent casein, (G-6) and 18 per cent casein (G-1) diets respectively. These values are identical to the protein regeneration which occurred in non-obstructed controls fed comparable diets.⁹

The group of rats subjected to common bile duct ligation, and one week later to partial-hepatectomy (*exper. 4*), fared better than expected. They ate well a 10 per cent casein, low fat diet (G-6), and were killed on the 14th day after partial hepatectomy.

⁶ Biliary obstructed rats fed 30 per cent fat diet had N intake = .33 gm; cal. intake = 101 cal.; cal. intake less fecal fat-excretion cals. = 78 cal.; N balance = -.29 gm. Biliary obstructed rats fed 3 per cent fat diet had N intake = .46 gm.; cal. intake = 139 cal.; and N balance = -.02 gm. (all per 100 gm. initial body weight).

⁷ Biliary obstructed rats fed 30 per cent fat diet with and without bile had fat intakes of 5.6 and 5.3 gm., and fecal fat excretions of 3.11 and 1.95 gm. respectively per 100 gm. initial body weight.

⁸ Biliary obstructed rats fed 3 per cent fat diet had a fat intake of 1.0 gm., and fecal fat excretion of 0.34 gm., while unobstructed pair-fed controls had fecal fat excretion of 0.14 gm. per 100 gm. of initial body weight.

⁹ Control values for diets G-2 and G-1 of 0.17 ± 0.01 and 0.37 ± 0.04 gm/100 gm. of initial body weight were obtained from data of Vars and Gurd (4).

They regenerated liver mass wet, 1.99 ± 0.43 gm., dry 0.50 ± 0.10 gm., and liver protein 0.31 ± 0.03 gm., with a nitrogen intake of 0.68 ± 0.16 gm., and a positive nitrogen balance of 0.18 ± 0.13 gm. Comparable unobstructed rats (4) regenerated liver mass wet, 1.98 ± 0.38 gm., dry 0.60 ± 0.13 gm., and liver protein 0.30 ± 0.02 gm., with a slightly greater nitrogen intake of 0.77 ± 0.09 gm., and a balance of $+0.24 \pm 0.06$ gm. all per 100 grams of initial body weight. These previously biliary obstructed rats also regenerated liver space with non-obstructed rats.

Lipid and glycogen determinations were done on all pooled livers of groups of rats at both operation and death. Lipid values at operation were constant at 6 to 7 per cent. At death they all remained the same or were less (3-4%). No livers were fatty in either the biliary obstructed or unobstructed rats, even when the diet contained 30 per cent fat. Glycogen values at operation varied from 6 to 10 per cent and at death were universally below 1 per cent in obstructed animals and in pair-fed controls, but were maintained around 6 per cent in *ad libitum*-fed controls.

Plasma proteins at death were all of a fairly uniform level of 5 gm. per cent, although rats which received higher protein diets tended to have slightly higher plasma protein values than did rats with a lesser protein intake.

Prothrombin percentage never reached critical levels in any of the rats. Biliary obstructed rats had levels of approximately 70 to 80 per cent while controls had 90 to 100 per cent. The icterus index of the jaundiced rats varied from 35 to 62 (normal 18).

Seventy-eight rats were subjected to common bile duct ligation with and without partial hepatectomy. Of these 14 could not be used either because of some associated pathology or death. The over-all mortality of the biliary obstructed animals was 15.4 per cent.

Grossly, all biliary obstructed livers developed increasing fibrosis depending on the duration of the obstruction. At 14 days, the livers were large, tawny in color and of a firm rubbery consistency. Several showed early fine nodularity, and pin point areas of necrosis were observed. The common bile ducts were tense, thin-walled and markedly dilated, containing from one to three cc. of usually pale watery bile, but occasionally thicker turbid bile. Adhesions were frequent, and often the duodenum was displaced by the distended choledochus, but no instances of intestinal obstruction were noted. Routinely the intestinal tract was inspected to ascertain the absence of bile staining. Occasionally in rats that died and in a few which were killed a massive lobar necrosis was observed in the livers, particularly where the distended common bile duct caused pressure of the liver against the rib margin or vertebral column. This distribution of necrosis was previously observed and reported by Estrada, Simpson and Vars (7) in their studies of gastric distention and liver damage. Data obtained from these livers were not included in the computation of the results. Two or three livers contained small hilar abscesses, invariably accompanied by wound infection. These also were excluded. No other organs of the body showed any abnormality, although in long standing biliary obstructed rats, the spleen always appeared slightly larger than in controls. Ascites was not observed.

Microscopically, the biliary obstructed livers showed a progressive inter and intralobular fibrosis, occurring predominantly in portal areas. Marked ductal pro-

liferation was present. The liver cells showed no evidence of fatty infiltration. They were frequently irregular with shrunken cytoplasm, no doubt partly due to their low glycogen content. Usually they stained pale, except for occasional cords of cells which stained darker. Tiny focal areas of necrosis were frequently observed in various stages of formation and repair. Small numbers of inflammatory cells were seen in these areas of necrosis but not elsewhere. The kidneys and spleen were also examined microscopically and showed no marked abnormality. Bile thrombi were seen in the kidneys; the spleens were congested and appeared to have increased fibrous tissue stroma.

CONCLUSIONS AND SUMMARY

Protein-depleted rats, subjected to partial hepatectomy and to simultaneous high ligation and division of the common bile duct, regenerated in the 14-day post-operative period liver mass and liver protein in excess of *ad libitum*-fed and pair-fed control rats subjected to the same procedures except for ligation and division of the common bile duct. After subtracting the amount of (liver) protein estimated to be due to bile duct proliferation and hepatic fibrosis, the parenchymal cell protein regeneration in biliary obstructed rats equalled that occurring in unobstructed pair-fed controls. Liver protein regeneration in biliary obstructed rats, as in unobstructed rats, was proportional to the protein intake in the post-operative period.

A high fat diet did not decrease liver protein production though it increased the negative nitrogen balance. The addition of bile to the high fat diet caused no material change in metabolism or liver regeneration. Jaundice per se in previously protein-depleted rats did not alter the nitrogen balance from that of unobstructed pair-fed rats. Counts of parenchymal cell mitoses at intervals following partial hepatectomy indicated active parenchymal regenerative efforts on the part of the biliary obstructed livers.

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EFFECT OF ECK FISTULA FORMATION, SIMPLE PORTAL OBSTRUCTION AND 'MEAT INTOXICATION' ON SERUM PHOSPHATASE AND DYE CLEARANCE OF ADULT DOGS

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THE general effects of depriving the liver of its portal blood supply by means of an Eck fistula (portal vein ligation proximal to the site of portacaval anastomosis) has been described by numerous investigators (1-3). It is commonly agreed that Eck fistula dogs ordinarily manifest anorexia and weight loss, frequently dying in an advanced state of cachexia, anemia and debility. Impairment in the ability of the Eck fistula dog to synthesize plasma proteins, hemoglobin (4) and bile salts (5), to store fluids (6) and destroy uric acid (7) have been reported. However, dye clearance studies reported by Bollman and Mann (7) failed to demonstrate definite impairment in liver function, although their comments indicate that an impairment could be demonstrated with large amounts of rose bengal. Portal obstruction without portacaval anastomosis results in less atrophy of the liver than occurs in the Eck fistula dogs and the animals remain in relatively good health (3).

The intolerance of Eck fistula dogs to meat has long been recognized (1), but Whipple *et al.* have shown (4) that Eck fistula dogs can be maintained in relatively good health for several years. It has not been reported that dogs with simple portal obstruction are susceptible to meat intoxication.

The literature pertaining to Eck fistula and simple portal vein obstructed animals contains little data with regard to dye clearance and essentially no information concerning serum phosphatase activity. Previous studies from this laboratory have shown that the rose bengal dye clearance and serum phosphatase tests can be used to demonstrate changes in hepatic function under certain circumstances. In the present study these tests were applied to Eck fistula, simple portal vein obstructed, and normal dogs, with and without meat feeding.

EXPERIMENTAL PROCEDURE

Medium to large (30-40 lb.) adult dogs of both sexes were used for this study. The postoperative diet routinely consisted of Pard, bread and milk. Initially the Eck fistula was formed according to the procedure described by Fishback (2). More recently a special clamp was devised to aid the formation of this anastomosis (8). Simple complete portal ligation was carried out in two stages. The second operation was usually 3 to 4 weeks after partial portal obstruction had been produced. Obstruction of the portal vein both with and without portacaval anastomosis was always just proximal to the point of entrance of the pancreatic vein into the main portal vessel. 'Meat intoxication' was produced by feeding 50 gm/kg. of ground raw lean horsemeat daily. Frequently it was necessary to force-feed the operated dogs, in which case the diet was more completely retained if fed in divided portions. The methods of estimation of serum phosphatase and rose bengal dye clearance have been described

previously (9). These tests were usually made at weekly intervals on each animal during the period of study, which varied from a few weeks to several months.

RESULTS

The effects of Eck fistula formation on the dye clearance and serum phosphatase of the dog are shown in the accompanying table. Without exception there was a definite decline in the dye clearance. This decline was apparent soon after the operation and usually progressed, reaching values in some instances that were only approximately one-third of the preoperative value. Following Eck fistula formation the serum phosphatase underwent a definite increase (see table). In some animals it reached values that were many times normal, while in other instances the rise was not so striking. Usually there was an inverse relation between the dye clearance and the serum phosphatase value.

Simple portal obstruction usually produced a slight decline in the dye clearance, accompanied by an increase in serum phosphatase (see table). The change from the normal values for these tests after simple portal obstruction was less striking than that after Eck fistula formation. The *t*-ratios indicate that the changes are significant after both operations.

'Meat intoxication' was produced in 11 Eck fistula dogs. The incidence of meat intoxication was increased by using the clamp in preparing the portacaval anastomosis. In every instance the appearance of the symptoms of 'meat intoxication' (ataxia, blindness, spasticity) was associated in Eck fistula dogs with an increase in serum phosphatase (see table). The individual increase varied considerably and the striking increase in one animal from 11 to 65 Bodansky Units lowered the *t*-ratio. Including this animal it is 2.54; excluding this animal, 3.5. However, both values indicate that there is little likelihood that the observed differences could be explained as due to chance variation (2% and 1%, respectively, 9a). In one instance a marked increase (88 units in 26 days) in serum phosphatase occurred in an Eck fistula dog during the period of meat feeding, but the dog remained in good health. In 7 out of 12 instances of 'meat intoxication' in Eck fistula dogs there was a striking associated decline in dye clearance, while in 5 other instances this change was relatively slight. The onset of 'meat intoxication' was often sudden; some dogs became moribund in 24 to 48 hours after the onset of symptoms. The average time required for 'meat intoxication' to occur was 9 days after the onset of meat feeding. The animals that rapidly became moribund showed the least change in liver function tests.

There were no consistent changes in the liver function tests during the time that the portal vein obstructed dogs were fed meat (see table). 'Meat intoxication' was produced in one of 5 simple portal vein obstructed dogs fed exclusively on horsemeat without significant changes occurring in the liver function tests. This animal manifested the classical symptoms of 'meat intoxication' after 7 days on meat and was twice presumed to be dying. However, it made a spontaneous recovery, resumed eating meat, and did not develop meat intoxication again even though the daily intake of meat was increased to 100 gm/kg. daily for 3 weeks.

Four normal dogs fed meat showed no change or a slight decline in dye clearance. Their phosphatase values remained within the normal range (see table).

TABLE I

CONDITION OF DOGS	DYE CLEARANCE ¹						SERUM PHOSPHATASE ²						Expt. period	Spread in days	
	No. of dogs	Aver.	S.D.	S.E.	Diff. of means	t ratio	No. of dogs	Aver.	S.D.	S.E.	Diff. of means	S.E. of diff.			t ratio
Eck fistula															
Before operation	24	106	10.9	2.21	61	3.4	22	2.3	1.2	.256	10.2	1.71	5.96	9-69	
After operation	24	45	15.8	3.24			22	12.5	7.84	1.67					
Portal obstruction															
Before ligation	6	114	13.3	5.36	34	7.4	7	1.8	1.10	0.41	4.6	1.25	3.71	40-119	
After ligation	7	80	13.3	6.01			7	6.4	3.1	1.17					
Eck fistula															
Before meat intoxication	12	51	11.4	3.31	17	4.23	12	6.6	3.0	.87	12.2	4.86	2.54	3-31	
During meat intoxication	12	34	9.38	2.7			12	18.8	16.0	4.65					
Portal obstruction															
Before meat diet	5	87	15.9	7.1	11	12.9	5	4.9	1.24	.55	1.6	2.48	.645	34-144 ⁴	
During meat diet	5	76	33.1	14.9			5	6.5	5.47	2.45				7-20 ⁶	
Normal															
Before meat diet	4	111	11.5	5.75	14	9.74	4	1.75	1.17	.59	.57	1.02	.56	15 ⁶	
During meat diet	4	97	14.7	7.35			4	2.32	1.15	.57				13-21 ⁶	

¹ = arbitrary units; ² = Bodansky units; ³ = days after Eck fistula operation; ⁴ = days after portal ligation; ⁵ = days required for meat intoxication to develop in Eck fistula dogs; ⁶ = days on meat diet.

DISCUSSION

The observations herein presented indicate a definite reduction in the rate of rose bengal excretion as a result of Eck fistula formation. The reduction was variable in amount, but the average reduction was to a value approaching half of the preoperative one. The lowest values for dye excretion were usually not obtained immediately after operation; therefore, it is reasonable to suppose that secondary changes occurring in the liver contribute to the loss of excretory function for the dye. The liver is reduced in size after Eck fistula formation, indicating that considerable parenchyma must be lost. In addition, an increase in liver fat may occur. Failure of others to demonstrate clearly the effect of Eck fistula formation on dye clearance was probably due to a difference in the technique used for demonstrating the rate of dye disappearance.

Simple portal obstruction also caused a definite reduction in the rose bengal dye clearance. However, the effect was less marked and less consistent than the reduction caused by Eck fistula formation. The difference between the two groups probably reflects the difference in the degree of reduction in hepatic circulation. Portal hypertension is produced by simple ligation of the vessels and this stimulates the development of a collateral circulation, part of which goes to the liver, as evidenced in the group herein presented by the enlargement of numerous small vessels entering the hilus of the liver. Formation of an Eck fistula with an adequate stoma does not lead to portal hypertension, as judged in this group by the absence of well-defined venous collaterals at the hilus of the liver. The fact that there is less atrophy of the liver in dogs with simple portal obstruction than in Eck fistula animals is further evidence that the former group has less reduction in venous blood entering the liver.

'Meat intoxication' in the Eck fistula dog frequently caused a further reduction in dye clearance. The average effect in the 12 instances reported was less definite than the results obtained in certain individual cases. In other instances 'meat intoxication' was not associated with any further decrease in the dye clearance. The results show that in slightly over half of the instances 'meat intoxication' produced a definite and rapid decline in the rate of removal of rose bengal from the circulation. This observation does not agree with that recently reported by Drill (10). Meat feeding in the simple portal vein obstructed dogs caused a significant decrease in dye clearance in only 2 animals.

An increase in serum phosphatase always resulted from Eck fistula formation. The magnitude of increase and the rate of change varied from animal to animal, but the tendency was the same in every instance. Probably there is a relation between the size and patency of the stoma and the postoperative rise in serum phosphatase activity. The serum phosphatase rise reached a maximum at varying times after Eck fistula formation and was frequently but not invariably associated with a minimum dye clearance in a particular animal. 'Meat intoxication' consistently caused a further increase in serum phosphatase in the Eck fistula dog. In some animals the inverse relation between the serum phosphatase and dye clearance (previously demonstrated in the protein-deficient dog, 9, 11), was apparent, whereas meat poisoning caused a definite change in serum phosphatase with little or no further decline in dye clearance in other animals. There are, however, interesting exceptions to both of these statements. For instance, the dog with simple portal obstruction that developed meat intoxication showed a slight decline in serum phosphatase at the time of 'meat intoxi-

cation.' An Eck fistula dog, which remained in good health during 26 days of meat feeding, showed a marked rise in serum phosphatase during this period with subsequent decline when the meat diet was discontinued.

There is a difference of opinion as to the significance of the increase in serum phosphatase that occurs in relation to liver disease. Some (12, 13) hold that the rise is the result of impaired excretion of the enzyme from the blood stream by the liver, whereas others (14, 15) maintain that the increase results from the liver phosphatase gaining access to the circulation in increased amounts. If the rise in serum phosphatase were simply due to a failure of hepatic excretion of phosphatase from the blood stream, its rise would always inversely parallel changes in dye clearance. There are numerous instances wherein this association does not seem to exist. It was also observed by Dameron and the author (16) that bile or jaundiced blood rich in phosphatase injected intravenously into bile fistula dogs failed to alter the phosphatase output in the bile, although the injected phosphatase disappeared from the circulation. In a previous publication (15) other experimental evidence was presented favoring the view that the increased serum phosphatase resulting from obstruction or injury to the liver largely originates within the liver itself rather than in the skeleton or elsewhere in the body.

Regardless of the origin of the serum phosphatase, its rise in the absence of extra-hepatic bile duct obstruction indicates that Eck fistula formation causes active damage to the liver and that this damage is aggravated by 'meat intoxication.' The fact that 'meat intoxication' contributes to disturbed hepatic function as measured by dye clearance and serum phosphatase may be taken as evidence that further failure of hepatic function is usually associated with 'meat intoxication' in the Eck fistula dog. The occurrence of 'meat intoxication' without changes in dye clearance or serum phosphatase activity in a dog with simple obstruction of the portal vein indicates that neither of these measurements is directly related to the hepatic failure that caused these symptoms.

SUMMARY

Eck fistula formation and simple portal vein obstruction have qualitatively the same effects upon serum phosphatase and rose bengal dye clearance. Eck fistula formation consistently reduced the rose bengal dye clearance and increased the serum phosphatase of adult dogs. 'Meat intoxication' regularly caused a further increase in the serum phosphatase and frequently caused a definite decline in the dye clearance of Eck fistula animals. However, meat feeding did not consistently produce any further change in the liver function tests of the simple portal vein obstructed dogs, although 'meat intoxication' was produced in one such animal. The foregoing evidence indicates that neither liver function test is an index of the hepatic failure that results in the symptoms of 'meat intoxication.'

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EFFECT OF IMPAIRED HEPATIC CIRCULATION ON PLASMA FREE AMINO ACIDS OF DOGS

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ECK fistula dogs fed a diet of raw horsemeat and water frequently exhibit signs of 'meat intoxication.' These include muscular rigidity similar to that seen in decerebrate animals and loss of senses, particularly vision. Some animals appear semi-stuporous, with ataxic gait and loss of righting reflexes. Animals with portal obstruction generally do not intoxicate upon the raw horsemeat diet. The literature contains no information concerning the effect of meat intoxication on the plasma concentration of the individual amino acids.

Early investigations by Van Slyke (1) indicated that during digestion there was a greater fall in the amino nitrogen content of the blood in its passage through the liver of the normal animal than through the entire remainder of the body. The reduction of blood supply to the liver by Eck fistula formation did not increase the blood amino nitrogen content of dogs, according to Whipple and Van Slyke (2). Acceleration of amino acid liberation in their animals by meat feeding or toxic tissue autolysis failed to elevate amino nitrogen levels.

The advent of microbiological procedures for the determination of individual amino acids has made possible the detailed study of these substances in animals with altered hepatic circulation. This report concerns the effect of Eck fistula formation on the plasma free amino acids of fasted dogs and the effects of meat feeding on dogs with portal obstructions and those with Eck fistulas.

EXPERIMENTAL

Surgical Procedures. Initially Eck fistulas were formed according to the procedure described by Fishback (3). More recently a special clamp was devised to aid the formation of this anastomosis (4). Simple complete portal ligation was carried out in two stages. The second operation was performed 3 to 4 weeks after partial portal obstruction had been produced by constriction of the portal vein to approximately half of its normal diameter just proximal to its junction with the pancreatic vein.

Diets. Dogs which were used for the determinations of normal ranges of amino acids were maintained on bread, cooked horsemeat and water. Animals with Eck fistulas were fed a bread-milk-Karo syrup ration. The meat diet for the production of intoxication consisted of raw horsemeat, 50 mg/kg. body weight per day.

Microbiological Determinations. Heparinized blood samples were centrifuged and the plasma treated according to the method of Hier and Bergeim (5). *L. arabinosus* 17-5 was used for the assay of tryptophane, leucine, isoleucine and valine. Histidine,

methionine, tyrosine, proline, arginine, glycine, cystine, serine and lysine were measured with *Leuconostoc mesenteroides* P-60. *Streptococcus faecalis* R. was employed in the assay of threonine and *L. casei* 7469 for phenylalanine. Assay media were dis-

TABLE 1. FASTING LEVELS OF PLASMA FREE AMINO ACIDS IN NORMAL DOGS

AMINO ACID	NO. ANIMALS TESTED	NORMAL RANGE	AVERAGE	AMINO ACID	NO. ANIMALS TESTED	NORMAL RANGE	AVERAGE
<i>μg/ml. plasma</i>				<i>μg/ml. plasma</i>			
Histidine.....	36	6.6-39.7	22.34	Arginine.....	12	6.0-52.5	24.9
Tryptophane.....	22	3.2-26.0	13.25	Threonine.....	21	11.4-48.3	25.9
Methionine.....	27	2.2-18.0	9.7	Phenylalanine...	13	8.2-22.5	14.5
Tyrosine.....	28	4.9-33.0	18.4	Cystine.....	13	9.1-23.3	16.4
Proline.....	17	7.5-34.2	17.2	Serine.....	13	4.8-18.6	11.3
Leucine.....	13	16.2-45.0	34.5	Glycine.....	10	17.8-39.5	27.2
Isoleucine.....	12	2.4-22.5	13.4	Lysine.....	7	22.5-41.6	30.8
Valine.....	15	15.0-37.5	27.2	Aspartic acid....	20	< 1	< 1

TABLE 2. CONSTANCY OF FASTING LEVELS OF FREE AMINO ACIDS IN DOG PLASMA

WEEKLY SAMPLES	ISOLEUCINE	LEUCINE	METHIONINE	TRYPTOPHANE	TYROSINE	VALINE
<i>μg/ml. plasma</i>						
<i>Dog 189</i>						
A	23.2	24.3	6.9	7.1	7.8	13.6
B	29.6	28.2	5.4	6.3	6.0	18.6
C	39.7	26.3	7.2	8.7	6.3	20.3
D	33.5	28.0	7.8	8.4	12.6	24.6
<i>Dog 186</i>						
A	37.8	23.6	7.9	6.5	3.9	22.5
B	36.0	19.4	7.8	7.1	4.5	19.5
C	22.8	17.4	6.6	9.3	3.6	20.4
D	30.8	22.5	7.8	8.3	6.3	22.7
<i>Dog 185</i>						
A	41.7	30.0	7.8	7.4	10.5	20.4
B		31.8	6.6	8.6	9.3	22.5
C	36.3	30.7	6.0	10.8	6.3	21.9
D	28.4	30.7	7.2	10.7	10.2	22.8
<i>Dog 182</i>						
A		30.0	6.9	6.9	8.4	29.6
B		30.0	7.2	7.6	7.5	22.2
C		30.0	8.7	8.1	9.9	23.4
D		33.7	8.1	9.5		33.3

pensed in 1 ml. amounts, and samples and water brought the final volume in the tubes to 2 ml. Each sample was assayed in duplicate at 3 levels. Standard curves were set up in triplicate for each assay. All data are derived from plasma obtained 12 or more hours after the last feeding, with the exception of that contained in table 5.

RESULTS

Table 1 provides a list of the amino acids studied in normal dogs. The ranges and averages obtained from this survey were employed for comparison with blood levels of dogs with hepatic insufficiency.

TABLE 3. FASTING LEVELS OF PLASMA FREE AMINO ACIDS IN DOGS WITH SIMPLE PORTAL OBSTRUCTIONS ON HORSEMEAT INTOXICATION DIET

DOG NO.	DAY OF DIET	OBSERVED CONDITION	HISTIDINE	TRYPTOPHANE	METHIONINE	TYROSINE	PROLINE	VALINE	ARGININE	THREONINE	PHENYLALANINE
38	0	Normal	18.0		7.5	8.2	10.5			25.4	
	13	Normal	21.0		7.5	11.1	9.6			12.5	
53	0	Normal	7.5	8.0	6.7	13.5	4.8				
	5	Normal	30.0	9.6	13.5	22.0	15.0				
	12	Normal	16.5	4.8	3.9	13.8	6.6				
50	0	Normal	15.6	9.3	7.5	35.1	12.3			45.0	
	5	Normal	42.9 ¹	12.0	14.2	36.6	22.2			37.5	
	7	Sick	45.6 ¹	10.8	21.7	41.1	30.6			48.7	
	12 ²	Very sick	24.0	7.5	4.2	18.0	0.0 ¹			28.0	
	20	Improved	18.0	7.1	6.5	15.0	9.0			17.5	
	24	Improved		9.9	14.7	21.0					
	28	Improved		3.0	13.6	12.0					
55	0	Normal		6.8	9.7	7.5		10.6	31.5		13.5
	6	Normal		7.5	12.7	11.4			60.0 ¹		24.4
	53	Normal		10.5	9.7	8.1			47.7		14.2
	62	Normal		14.3	9.6	12.0		30.0			18.4
	67	Normal		10.8	20.6	8.4		16.2			15.0
73	0	Normal	11.2	6.6		11.4	17.7		52.5		17.5
	6	Normal	9.0	8.1		11.4	16.5		37.5		12.7
	53	Normal				14.4	16.8		52.5		16.8
	62	Normal		15.0		11.4	10.9				12.5
	67	Normal		15.9		12.9	16.5				12.9
98	0	Normal		8.3	9.3	9.0		16.8	37.5		13.8
	6	Normal		9.0	15.0	9.0		17.8	43.5		10.7
	53	Normal		15.0	11.1	6.0		32.8	72.3 ¹		19.5
	62	Normal			9.9	7.5		30.0			16.8
	67	Normal		11.3	10.2	12.0		33.8			15.7

¹ Outside of normal range of plasma values for dogs. ² Intoxication too severe for feeding on 11th day.

To determine the constancy of fasting levels in a given animal, samples were obtained from 4 dogs during 4 consecutive weeks. Table 2 shows the degree of variation found in the levels of 6 amino acids. Methionine and tryptophane were the least variable; isoleucine, the most. The results of this experiment indicate that there is

TABLE 4. FASTING LEVELS OF PLASMA FREE AMINO ACIDS IN ECK FISTULA DOGS MAINTAINED ON HORSEMEAT INTOXICATION DIET

DOG NO.	DAY OF DIET	OBSERVED CONDITION	HISTIDINE	TRYPTOPHANE	METHIONINE	TYROSINE	PROLINE	ARGININE	THREONINE	PHENYLALANINE	CYSTINE	LEUCINE	ISOLEUCINE	VALINE	LYSINE	GLYCINE	SERINE
<i>μg/ml. plasma</i>																	
62-I	0	Normal	24.6	14.4	7.5	14.2	19.2		20.7								
	11	Intoxicated	33.9	24.0	17.8	37.5	17.6		21.2								
62-II	0	Normal	18.3	10.0	6.0	30.3	12.3	33.3	46.8	15.7							
	5	Normal	37.5	10.8	14.5	37.5	23.1		36.5								
	10	Sick	37.5	12.0	17.5	37.6	21.5	51.9	41.2								
	20	Intoxicated		12.3	21.3 ¹	42.6 ¹		47.4	37.5	75.0 ¹							
51	0	Normal	25.0	10.5	12.0	16.4	25.0		25.8								
	28	Normal	26.0	12.3	11.1	24.3	15.0		15.3								
43	0	Normal	23.0	13.2	3.9	6.3			28.2								
	20	Normal	24.2	13.8	12.3	26.0			13.6								
61	0	Normal			13.9	38.7		38.0									
	8	Terminal			49.5 ¹	72.0 ¹		75.0 ¹									
85	0	Normal	18.6	10.1	11.2	28.2	19.8	6.0	34.5	17.1							
	6	Intoxicated	39.0	8.4	9.0	26.2	19.5	37.5	34.5	22.1							
74	0	Normal	22.2	19.5	14.4	39.6	22.5		33.7								
	2	Intoxicated	54.0 ¹	10.8	26.7 ¹	48.9 ¹	30.0		61.9 ¹								
76	0	Normal	11.1		9.9	27.3	9.9	37.5	20.4	9.2							
	5	Sick	24.9	7.8	8.5	27.0	13.8	30.5	28.0	12.0							
	7	Terminal	31.5	8.1	15.7	31.5	16.2		33.7	22.8							

relatively little fluctuation from week to week in 5 of 6 plasma-free amino acids of normal dogs on a bread and cooked-meat diet. It was considered necessary to establish this point in order to evaluate the results on control animals.

Plasma free amino acids were measured in 5 dogs before and after Eck fistula formation. The post-operative samples were taken 24 to 30 days after surgery. All 5 animals showed a slightly reduced plasma cystine, while 4 of 5 had less leucine and 3 of 5 less serine after the operation. Arginine was slightly reduced in the 2 animals tested. Tryptophane exhibited little fluctuation. Histidine was elevated slightly in 2 of 3 dogs, remaining the same in the third animal. None of the elevated or depressed levels were outside the ranges established previously as normal.

Three normal dogs and 13 dogs with Eck fistulas were placed on a raw horsemeat diet. The normal dogs showed no signs of poisoning even after 21 days. Eleven of the 13 with Eck fistulas developed signs of meat intoxication 2 to 11 days after the beginning of meat feeding. Eight of the 11 died. *Dog 62* was employed in 2 successive experiments and developed severe intoxication each time.

Six dogs with simple obstructions were included in the feeding experiments. Only one dog of this group became ill. He was noticeably sick on the 6th day of diet and on the 7th day appeared moribund. Meat was continued, by force when necessary, except on the 7th and 11th days when the animal was thought to be dying. The dog survived and resumed eating meat spontaneously, even when the ration was increased to 100 gm/kg. The normal dogs on the horsemeat diet showed decreased fasting levels of plasma free histidine, methionine, arginine, lysine and glycine. Tyrosine, isoleucine and serine remained fairly constant while tryptophane rose slightly in the one animal studied for this compound.

Tables 3 and 4 contain the results of amino acid assays on the plasma of the remaining dogs. They also show the length of time the animals had been on the raw horsemeat diet and their status at the time the blood samples were drawn. Two of the dogs with simple portal obstruction were found to have arginine levels above the upper limit of normal although they exhibited no sign of intoxication. *Dog 50*, which manifested the typical appearance of an intoxicated animal, showed a rise in amino acid levels at the time when poisoning became apparent, followed by a generalized reduction. In some cases the amino acids dropped below the levels obtained before meat feeding was begun. Proline was not detectable in this animal at the height of the disorder. Three of the 4 dogs studied for histidine showed elevated plasma levels. In the case of *dog 50* histidine was above normal.

Dogs 51 and *43* had Eck fistulas but did not become intoxicated on the raw-meat diet. They showed a rise in tyrosine and a drop in threonine levels after 28 and 20 days, respectively, on the diet. The Eck fistula dogs which developed meat intoxication differed from the normal in that most of the amino acids were elevated. Six of 8 dogs showed increased histidine. Six of 7 had elevated tyrosine levels. Seven of 7 showed increased methionine, 4 of 5 increased leucine and 3 of 4 elevated glycine levels. Arginine rose in 4 out of 5 dogs. Cystine and phenylalanine increased in all dogs studied for these amino acids, and serine and proline rose in 3 of 5 animals. Valine, tryptophane, isoleucine, lysine and threonine gave inconsistent results. Six of the 11 intoxicated dogs had fasting plasma levels above normal for 2 to 5 amino acids.

Table 5 shows the rise in plasma free amino acids following a meal of raw horse-meat. These data are the only ones submitted which do not represent fasting levels. The differences between values before and after feeding are greater for the Eck fistula animals than for the normal in all cases except tryptophane. Seven amino acids in the intoxicated and 6 in the unintoxicated Eck dog were present after feeding in amounts higher than the upper limit of the normal fasting levels. Only tryptophane and valine were in excess in the control. Three amino acids in the normal dog and 5 in the Eck were approximately doubled after feeding.

DISCUSSION

The findings described above indicate that Eck fistula formation and the resultant decreased circulation of blood through the liver does not in itself elevate the fasting free amino acid levels in plasma of dogs fed a diet consisting of bread and meat.

TABLE 5. EFFECT OF A 50 GM/KG. RAW HORSEMEAT MEAL ON PLASMA FREE AMINO ACIDS IN DOGS

AMINO ACID μg/ml. plasma	DOG 51 BEFORE	(ECK FISTULA) AFTER ²	DOG 61 BEFORE	(INTOX. ECK) AFTER	DOG 63 BEFORE	(NORMAL) AFTER
Threonine.....	14.7	34.9	14.8	27.5	15.1	28.0
Tryptophane.....	14.1	19.4	19.7	37.5 ¹	11.1	37.5 ¹
Methionine.....	14.7	22.9 ¹	13.8	27.6 ¹	8.2	16.8
Tyrosine.....	28.0	43.7 ¹	38.7	70.9 ¹	10.5	16.6
Proline.....	15.7	38.3 ¹	25.8	38.4 ¹	23.2	31.0
Histidine.....	28.0	69.0 ¹	23.3	64.5 ¹	18.5	28.4
Arginine.....	21.6	42.6 ¹	48.0	63.0 ¹	42.6	47.4
Phenylalanine.....	16.8	34.1 ¹	9.9	12.6	11.2	11.4
Valine.....	16.2	17.7	24.6	41.5 ¹	33.7	45.0 ¹
Leucine.....			38.7	41.4		
Isoleucine.....			12.2	18.0		

¹ Above upper limits of fasting range. ² Blood samples were taken 5 hours following the meal.

This is in agreement with the previous work of Whipple and Van Slyke on blood amino nitrogen. Meat feeding, however, was associated with a demonstrable difference between Eck fistula and normal animals. The former exhibited increased fasting levels of several amino acids during meat intoxication. Blood levels 5 hours after feeding also were shown to be higher in the dogs with Eck fistula. The 5-hour period was selected in view of the report of Van Slyke and Meyer (6) that blood amino nitrogen was approximately doubled at this time following a meal of raw beef.

Dogs with obstructions of the portal vein ordinarily have a greater tolerance for the raw meat diet than has the Eck fistula animal. Dog 50 of our series is an exception in that this animal developed unmistakable signs of meat intoxication. The histidine content of the plasma was elevated, as in the majority of other animals with meat intoxication.

The rise in plasma free amino acids in meat-intoxicated dogs is probably an indication of the decreased deamination which results from loss of the portal blood supply to the liver. The alterations in dye clearance and serum phosphatase (7) are further evidence of impaired liver function in the Eck fistula animal. Whether or not

elevated levels of specific amino acids are in themselves responsible for the intoxicated condition of the animals might be determined by feeding or injection studies with the compounds which were shown to increase significantly.

SUMMARY

Data have been compiled on fasting levels of 16 free amino acids in the plasma of normal dogs. Five of 6 amino acids studied at intervals in the same animals were shown to be relatively constant. Dogs with simple portal obstructions, fed a diet of raw horsemeat, showed variable alterations in amino acid levels. One of 6 dogs in this series became intoxicated but recovered. Normal dogs on the same diet showed decreases in several amino acids. The formation of an Eck fistula did not result in elevated plasma free amino acids. Eleven of 13 dogs with Eck fistulas showed signs of meat intoxication on a diet of raw horsemeat. During intoxication they exhibited increased plasma free histidine, tyrosine, methionine, leucine, glycine, arginine, cystine, phenylalanine, serine and proline. The 2 dogs which did not intoxicate showed an elevated plasma histidine and a decreased threonine. Other amino acids were relatively constant, even after 20 days on the diet. Both normal and Eck fistula animals showed a definite rise in the plasma free amino acids after a meal of raw horsemeat. The rise in levels of 6 amino acids was greater in Eck fistula dogs than in the normal control.

The authors wish to acknowledge the technical assistance of Miss Margaret Griesser.

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BROMSULPHALEIN REMOVAL RATES DURING HYPOTHERMIA IN THE DOG^{1, 2}

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IT IS generally agreed that a depression in body metabolism, as indicated by a reduced oxygen consumption, is usually found in animals with a rectal temperature of 25° C. or lower. Above this level a particular function may show a depression or an elevation. Dill and Forbes (1) have shown that the total energy exchange in prolonged hypothermia in humans with various subnormal body temperatures down to 25.5° C. may be two and even three times the basal normal. In dogs undergoing progressive cooling Penrod (2) has found an increased oxygen consumption that reaches a peak at about 28° C. rectal temperature. It is concluded in both of these experiments that the oxygen consumption is roughly proportional to the degree of shivering. Rosenhain and Penrod (3) report a progressive fall in cardiac output of dogs after 35° C. At this temperature and above, it may be increased, although the pulse rate begins to fall approximately linearly soon after the onset of cooling.

It was thought that a study of the body's ability to remove a foreign substance such as bromsulphalein might yield some information concerning the activity of the liver and the other removal sites of the dye during early hypothermia.

METHOD

In normal mongrel dogs moderately anesthetized with sodium pentothal, bromsulphalein (BSP)³ removal rate was measured using a single intravenous dose of 5 mg/kg. One to 3 days later the same dogs were again anesthetized and immersed in an iced bath (2-5° C.). The animals were divided into *groups A* and *B*. BSP removal was again measured, the injection being made when the rectal temperature reached 35° C. in *group A* and 30° C. in *group B*. Since these measurements were being made coincident with others, it was not feasible to keep the rectal temperature at a constant level. The temperatures continued to fall at a rate of approximately 0.17° C. per minute during the test.

Another series of 5 normothermic dogs was given 2 BSP tests under sodium pentothal at an interval of 24 hours in order to be sure that the brief interval between

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² This material was presented in part at the 1949 meeting of the Federation of American Societies for Experimental Biology, Detroit, and appeared in abstract form in *Federation Proc.* 8: 17, 1949.

³ The bromsulphalein was kindly supplied by Hynson, Westcott and Dunning, Inc.

tests and the anesthesia had no influence on the marked changes observed during hypothermia.

In the hypothermic dogs the dye injection was given via a carotid artery in order to insure adequate mixing with the circulating plasma. Blood samples for BSP concentration analysis were obtained from an intravenous catheter placed in the right side of the heart where there was a more active flow as well as more thorough mixing of venous blood than in the extremities of the cooled animals.

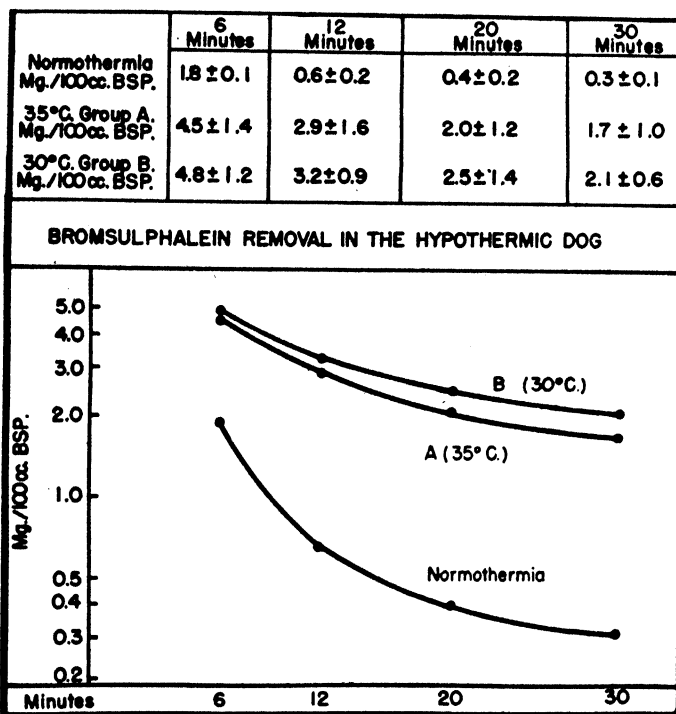


Fig. 1. AVERAGE BSP REMOVAL RATES of 16 anesthetized normothermic dogs, 7 dogs at 35° C (A), and 10 dogs at 30° C. (B). Ordinate: plasma BSP concentration plotted logarithmically. Abscissa time plotted linearly.

Blood samples were obtained at 6, 12, 20, and 30 minutes after the BSP injection in all animals. Plasma BSP determinations were made with a Coleman Jr. spectrophotometer⁴ using the technique described by Bradley *et al.* (4). All rectal temperatures were recorded by means of a Leeds and Northrup 'Speedomax' recorder.

RESULTS

Figure 1 summarizes the observed data. In the anesthetized normothermic control tests the removal was found to be prompt and rapid, not differing appreciably

⁴ This method is accurate to 0.1 mg/100 cc. Most errors will be on the low side of the true concentration. Hemolyzed or turbid samples will falsely show even lower BSP concentrations and hence are not used.

from that of conscious animals and in agreement with the results of other investigators. (BSP concentration 30 minutes after injection was 0.2 ± 0.1 mg/100 cc. and 0.3 ± 0.1 mg/100 cc. in 13 conscious and 16 anesthetized dogs respectively.) As can be seen from the accompanying graph, however, the percentage disappearance rate was not found to be constant as was the case in most of the humans examined by Ingelfinger *et al.* (5). The most rapid disappearance occurred during the first 12 minutes after injection followed by a more gradual decline in dye concentration so that by 30 minutes only a small fraction of the original amount of BSP was present.

The retarded removal rates in the two groups of hypothermic animals roughly paralleled each other with the colder dogs showing a greater retention and a slower rate. In a few animals blood samples were obtained one hour after the dye injection, and during the last 30 minutes only a very small additional amount of BSP was removed.

DISCUSSION

The exact mechanism for the BSP retention in hypothermia is not known. Two possible explanations are: 1) Depression of the metabolic activity of the removal sites, and 2) reduction in hepatic blood flow. Fuhrman and Field (6) have shown that there is a marked depression of the metabolism of rat kidney cortex slices and brain tissue as measured by their oxygen consumption and anaerobic glycolysis. The temperatures used by them (0.2° C.) however, were far more severe than those of these experiments.

The presence of shivering with increased oxygen consumption demonstrates that the metabolism of certain body cells is frequently elevated during early hypothermia with a peak oxygen consumption at about 28° C. rectal temperature. The glycogen stores of the liver are materially depleted indicating some increase in at least one of the functions of this organ (7).

Since the liver is believed to be the chief site for the removal of BSP, a reduced hepatic blood flow could be largely responsible for the slower removal rates. Mendeloff *et al.* (8) have found a decrease in BSP removal rates in normal humans during exercise. An immediate decrease in BSP removal has been found by Culbertson *et al.* (9) when the liver blood flow is diminished by the vascular adjustments that occur when a patient is tilted from a recumbent position to an upright one. These studies indicate a close relationship between BSP removal and total hepatic blood flow. However, cardiac output studies (3) on hypothermic dogs do not indicate that the total blood flow is materially reduced in the 35° C. range, but by 30° C. it is reduced by some 23 per cent, a figure in line with the reduced BSP clearance found. At this time the relative importance of diminished cellular activity and decreased blood flow in BSP retention cannot be decided. If the liver shares in the general reduction in cardiac output found after 35° C., it would appear that this factor is the more important one.

SUMMARY

Plasma BSP removal rates were measured on 7 dogs at 35° C. and 10 dogs at 30° C. rectal temperature. Both series of animals showed a marked retention and a slowed removal rate. The disappearance rate curves roughly paralleled each other with the 30° C. group showing a greater retention and a slower removal rate. De-

creased cellular activity or reduced hepatic blood flow or both may be responsible for the retarded removal rate.

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INFLUENCE OF GRADED ARTERIAL PRESSURE DECREMENT ON RENAL CLEARANCE OF CREATININE, P-AMINOHIPPURATE AND SODIUM¹

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IN A previous study designed to establish the relationship of renal blood flow to arterial perfusion pressure in the intact kidney of the dog, it was demonstrated that due to apparent renal autonomy, blood flow was maintained despite considerable decrease in perfusion pressure (1). This resulted in a curvilinear pressure-flow relationship convex toward the pressure axis. Since direct blood flow measurements only were made in this study, no conclusion could be drawn with regard to the behavior of the renal arterioles in this mechanism. Forster and Maes (2) studied the effects of elevation of mean arterial blood pressure on the clearance of creatinine and p-aminohippurate (PAH) in rabbits whose kidneys had been denervated and whose adrenal glands had been demedullated. They found that when blood pressure was elevated by neurogenic mechanisms resulting from clamping of the carotid arteries that these clearances remained remarkably constant. This apparent constancy of glomerular filtration rate and effective plasma flow appeared to result from increase in afferent arteriolar resistance.

In the present study, the effect of graded decrease in arterial blood pressure on the clearance of PAH, taken to measure effective plasma flow, and on creatinine clearance, measuring glomerular filtration rate, was studied in dogs. In addition, the reduction in glomerular filtration which accompanied the decrease in arterial pressure afforded an opportunity to examine the effect of reduced sodium load on the renal mechanism for sodium excretion. This was of particular interest because of the phenomenon of sodium retention accompanying the reduction in glomerular filtration rate and effective plasma flow noted in congestive heart failure (3, 4).

METHODS

Female dogs averaging 16.5 kg. in weight (range, 11.5 to 23.5) were used. They were anesthetized with 30 mg/kg. of pentobarbital sodium administered intravenously. The left kidney and dorsal aorta were exposed by a dorsal retroperitoneal approach. The left ureter was catheterized so that the tip of the catheter lay within the renal pelvis; the length of the catheter was kept to a minimum so that the dead space of the collecting system was kept to a negligible volume. With the exception of several of the earlier experiments, the right kidney served as a control. Its urine was collected by means of an indwelling bladder catheter.

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Arterial pressure decrement to the left kidney was produced by a tourniquet around the dorsal aorta just between the right and left renal arteries, made possible by the higher origin of the right renal artery. Gradual occlusion of the aorta by the tourniquet thus decreased arterial inflow pressure to the left kidney but kept the blood supply to the right kidney reasonably constant. Carotid mean blood pressure was taken as the index of arterial pressure to the right (control) kidney, and the femoral mean blood pressure was taken as the index of arterial pressure to the left (experimental) kidney. The validity of the latter procedure was tested in 3 dogs by introduction of a long cannula into the abdominal aorta via the femoral artery, ligated in position just behind the axis of the left renal artery. The results of these experiments were in accord with those in which renal arterial inflow pressure was measured via the femoral artery.

The plan of each experiment was to follow a pair of control periods with four stages of graded arterial pressure decrement, with two consecutive urine collection periods at each level, followed by return of arterial pressure to control levels with two final recovery urine periods. Adequate discard periods were observed between each level of arterial pressure, with longer periods during stages of low urine flow. To insure adequate urine volumes, all animals were hydrated with 200 to 300 cc. of 0.9 per cent saline prior to the initial urine period, and a moderate amount of mannitol was included in the infusion fluid containing creatinine and PAH so that about 12 mg/min/kg. of body weight were given following a 5-gm. priming dose. Constant infusion was obtained by use of a mercury pump. Control urine flow averaged 2.0 cc/min. for the left kidney. Bloods were taken before and after each pair of urine periods, and interpolated values corrected for emptying time were used for calculation of the clearances.

The method of Smith *et al.* (5) was used for PAH analysis. Plasma PAH determinations were done on CdSO_4 filtrates. Creatinine was measured by the alkaline picrate method (6). Sodium tungstate filtrates were used for plasma creatinine determinations. All analyses were made in duplicate. Sodium was analyzed with a Perkin-Elmer model 18-A flame photometer² on diluted urines and plasmas; in some cases, trichloroacetic acid filtrates were used for plasma sodium analysis. No difference from direct plasma analysis was noted. When sodium clearances were calculated, a plasma sodium correction for the Donnan effect was made by the factor: KP/W , in which $\text{K}_{\text{Na}} = 0.925$, and W (percentage of water in the plasma) was taken as 0.94.

RESULTS

Effect of Graded Arterial Pressure Decrement on Clearance of PAH and Creatinine. Ten animals are included in this series. In three earlier experiments control clearances on the right kidney were not made simultaneously with the experimental changes produced in the left kidney by decreased arterial pressure, hence systemic factors which might alter clearances could not be detected. Although these three experiments are in approximate agreement with those done later, the emphasis of this section will

² We are indebted to Dr. Viola Startzman, Dept. of Pediatrics, for use of the Perkin-Elmer flame photometer.

be placed on seven experiments in which both right and left kidney clearances were followed simultaneously.

A representative experiment is illustrated in figure 1. This experiment is particularly instructive because systemic factors which might influence renal blood flow appear to be absent during the 3-hour duration of the experiment, as evidenced by the constancy of the control clearances. Thus it can be concluded that alterations

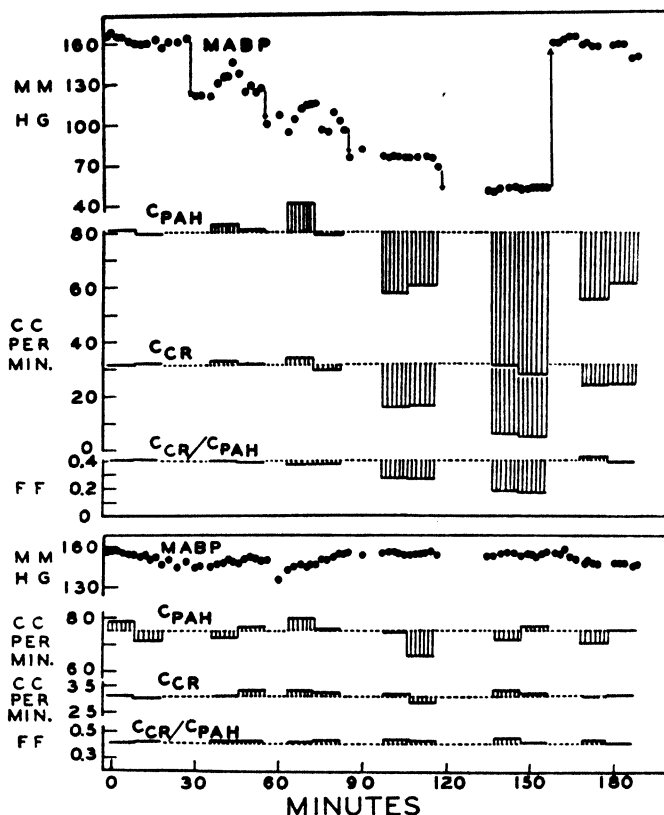


Fig. 1. EFFECT OF GRADED ARTERIAL PRESSURE decrement on renal clearance of PAH and creatinine, and on filtration fraction. *Upper*: experimental (left) kidney. *Lower*: control (right) kidney. Dashed lines in all cases designate the control data averages.

noted in the experimental kidney clearances must be specifically due to intrarenal changes resulting from the influence of decreased arterial pressure. The control clearances of PAH and creatinine, together with the filtration fraction, appear in the lower part of the figure in relation to the control arterial pressure.

In the upper part of figure 1 it is seen that as arterial pressure is progressively decreased from a mean of 162 mm. Hg through stages averaging 127 mm. and 104 mm. respectively, there are no significant changes in PAH and creatinine clearance, and the filtration fraction remains constant. At the level of 73 mm. Hg, however, clearances begin to decrease noticeably, and are significantly lower at a level averaging

50 mm. Hg. During the latter stages, the clearance of creatinine falls somewhat more rapidly than that of PAH, with the result that the filtration fraction decreases, a finding typical of all experiments. With release of the tourniquet, clearances recover to 78 per cent of the control kidney values. (In all experiments, average recovery for PAH clearance was to 89 per cent of the control kidney, and to 81 per cent of control for creatinine clearance.)

Systemic factors were frequently found to be operative which tended to decrease clearances somewhat during long experiments. In order to correct for this trend the experimental (left) kidney clearances are presented as a ratio to the control (right) kidney in figure 2 for all experiments. Each symbol in the figure represents the average of two consecutive urine collection periods. It is evident that the PAH clearance is well maintained as pressure is decreased to about 100 mm. Hg, then decreases rapidly with further decreases in pressure. Creatinine clearance is maintained to about 120 mm. Hg, then decreases somewhat more rapidly than the PAH clearance, resulting in decrease in the filtration fraction. At about 60 mm. Hg, all clearances rapidly approach zero.

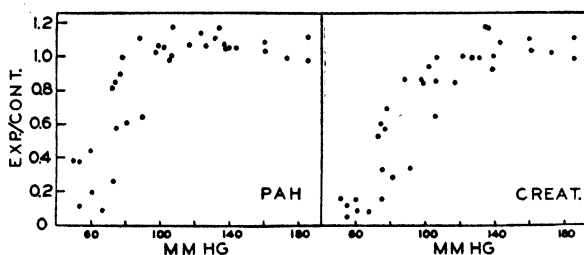


Fig. 2. EFFECT OF DECREASED ARTERIAL PRESSURE ON PAH and creatinine clearance expressed as a ratio to the control kidney. Each point is the average of 2 consecutive urine periods. Data are from 7 experiments.

Changes in Regional Renal Vascular Resistance Resulting From Graded Arterial Pressure Decrease. Changes in afferent arteriolar resistance (R_A), efferent arteriolar resistance (R_E), post-arteriolar resistance (R_V), and total renal resistance were analyzed by means of Lampport's equations (7).³ The same experiment graphically presented in figure 1 is used to exemplify the trend of resistance changes in figure 3. In the upper half of the figure PAH and creatinine clearance are given in cc/min/gm.

³ Calculation of regional renal vascular resistance in mm. Hg/cc/min. was made according to the following original equations of Lampport (7), with minor modifications cited below:

$$R_A = (P_M - P'_0 - 23 \text{ Hc} - 20)/\text{HD}; R_E = (1 - 0.47F)(P'_0 - P_0 - 23 \text{ Hc} + 10)/\text{HD}; R_V = (P_0 - P_V + 20)/\text{HD}. \text{ Total Renal Resistance} = R_A + R_E + R_V.$$

P_M = mean arterial blood pressure; P_0 = osmotic pressure of plasma protein, with protein concentration of plasma taken as 6 gm/100 cc.; P'_0 = osmotic pressure of plasma protein after glomerular filtration; P_V = renal vein pressure; Hc = hematocrit; HD = effective blood flow; F = filtration fraction.

The minor modifications employed in our calculations were to circumvent the spurious negative resistance values observed by Lampport (7) at substantially decreased arterial pressures. One modification substituted a yield pressure of 14 mm. Hg (1) for the value of 20 mm. Hg employed by Lampport, with an adjustment for nonlinearity for values below 80 mm. Hg arterial pressure. A second minor modification was to make intracapsular pressure proportional to glomerular filtration rate, instead of utilizing a constant value of 10 mm. Hg.

of kidney as related to arterial pressure. Here each point is the average of two consecutive clearance periods, and the trend of results is indicated by curves of best

Fig. 3. CHANGES IN REGIONAL RENAL VASCULAR RESISTANCE resulting from graded decrease in arterial pressure. *Upper:* changes in PAH and creatinine clearance in cc/min/gram for the same experiment shown in fig. 1. Each point is the average of 2 consecutive urine periods. *Lower:* R_{total} is total renal vascular resistance in mm. Hg/cc/min. R_A , afferent arteriolar resistance; R_V , postarteriolar resistance; R_E , efferent arteriolar resistance.

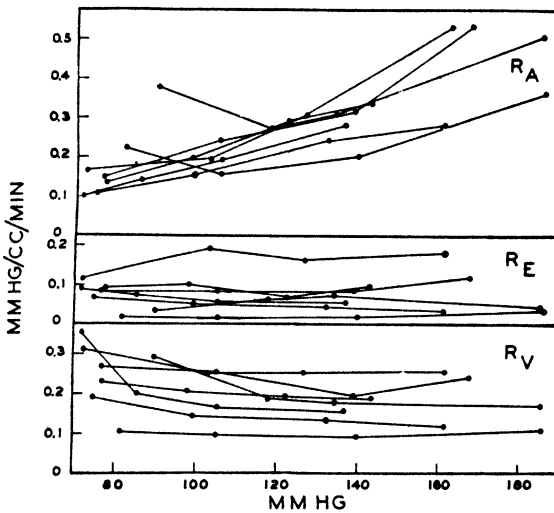
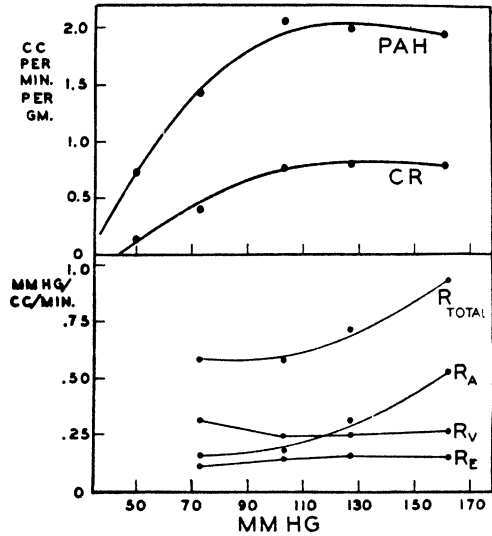


Fig. 4. RESISTANCE CHANGES for experiments shown in figure 2. Each point is the average of 2 consecutive urine periods. (Because of inconsistencies resulting from the small clearance values, calculations for the lowest pressure range are not included.)

fit for the experimental points. The maintenance of clearance during early stages of pressure decrement is obvious.

In the lower half of the figure appear the trends of resistance changes in the kidney. Total renal vascular resistance shows a gradual decrease through the range 162 to 104 mm. Hg, then remains constant to 73 mm. Hg. The decrease in total

resistance appears to be entirely attributable to decrease in afferent arteriolar resistance (R_A). R_x and R_v show no significant changes, although R_v may increase a bit at lower pressures. (The data for the lowest clearance figures are omitted in the resistance calculations because small absolute errors in the clearance data give large percentile errors in calculation.)

The conclusion that the constancy of plasma flow and glomerular filtration rate in the earlier stages of arterial pressure decrement is due to decrease in afferent

TABLE 1. REPRESENTATIVE EXPERIMENT SHOWING EFFECT OF DECREASE IN GLOMERULAR FILTRATION ON RENAL CLEARANCE OF SODIUM

(All data for one kidney only: weight 43 gm. Female dog, 13 kgm. body weight.)

PERIOD	MAP	FILT. RATE	SODIUM						
			Plasma conc. ¹	Load	Urine conc.	Urine vol.	Excreted	Reabsorbed	UV/P
	mm. Hg	cc/min.	mM/l.	mM/min.	mM/l.	cc/min.	mM/min.	mM/min.	cc/min.
1	141	39.4	134	5.27	45.2	2.25	0.102	5.17	0.76
2	146	45.3	136	6.13	64.0	2.00	0.128	6.00	0.94
Av.				5.70			0.115	5.58	0.85
3	124	35.9	140	5.05	113.0	1.10	0.124	4.93	0.89
4	121	32.2	142	4.62	77.4	0.95	0.074	4.54	0.52
Av.				4.83			0.100	4.73	0.70
5	99	23.3	138	3.23	20.9	0.50	0.010	3.22	0.0755
6	98	33.3	133	4.58	19.6	0.85	0.017	4.56	0.124
Av.				3.90			0.014	3.89	0.100
7	81	30.5	133	4.05	3.04	0.80	0.002	4.05	0.017
8	74	21.8	136	2.96	4.34	0.60	0.003	2.96	0.018
Av.				3.50			0.0025	3.50	0.017
9	66	8.3	141	1.17	0.00	0.30	0.000	1.17	0.000
10	55	4.0	142	0.58	0.00	0.20	0.000	0.58	0.000
Av.				0.88			0.000	0.88	0.000
11	145	33.0	142	4.70	23.0	1.30	0.030	4.67	0.20
12	138	35.7	142	5.07	39.5	1.40	0.057	5.01	0.40
Av.				4.89			0.043	4.85	0.30

¹ Plasma Na is corrected for the Donnan effect by the factor KP/W , in which $K_{Na} = 0.925$, and W (% of water in plasma) is taken as 0.94.

arteriolar resistance is confirmed in the combined data shown in figure 4. Note here again the general downward trend of R_A and the relative constancy of R_x and R_v .

Effect of Reduced Glomerular Filtration Rate on Renal Clearance of Sodium. In 5 animals gradual reduction in glomerular filtration rate by aortic occlusion was employed to study the effects of reduced sodium load to the renal tubules. Table 1 illustrates a typical experiment. During the control periods the average sodium load is 5.7 mM/min/kidney. Urinary excretion averages 0.115 mM/min. and the plasma clearance of sodium averages 0.85 cc/min. With reduction of load to 4.83 mM/min. urinary excretion decreases to 0.10 mM/min. and clearance to 0.70 cc/min. During the next stage in reduction of glomerular filtration rate, load is diminished

to 3.90 mm/min., urinary excretion is markedly reduced to 0.014 mm/min. and clearance decreases to 0.10 cc/min. as tubular reabsorption of filtered sodium becomes almost complete. This trend is continued in the remaining stages of reduced filtration rate. With restoration of arterial blood pressure and a return of filtration rate toward control values, urinary sodium excretion returns to 0.057 mm/min. and clearance 0.40 cc/min. during the last period when load is 5.07 mm/min.

The relationship of glomerular filtration rate to sodium excretion for all experiments is summarized in figure 5. This shows that sodium excretion diminishes as glomerular filtration rate is decreased from the control average of 40 cc/min/kidney, and that in a range 20 to 30 cc/min. urinary excretion is almost entirely abolished as tubular reabsorption of sodium becomes almost complete at reduced loads.

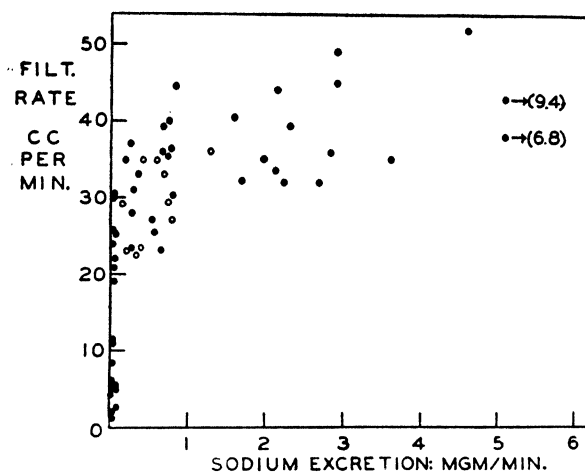


Fig. 5. RELATIONSHIP OF SODIUM EXCRETION to glomerular filtration rate (5 experiments). Open circles: values obtained after return of arterial pressure to normal after experimental changes. It is observed that these data are in the range of normal. Data are for one kidney only.

The relationship of sodium load to urinary excretion and tubular reabsorption is summarized for all experiments in table 2. Here it is revealed that the most pronounced decrease in excretion occurs at the second level of reduced glomerular filtration rate (average, 32 cc/min.), where average excretion is only 0.0173 mm/min. at a load of 4.3 mm/min. At this stage, reabsorption of filtered sodium is 99.5 per cent complete, as compared with 97.3 per cent during the control periods.

DISCUSSION

In connection with the previous work (1) employing direct blood flow measurement in analysis of pressure-flow relationship of the kidney, speculation was raised concerning the possibility that changes in blood viscosity resulting from changes in glomerular filtration might be the basis for the apparent renal autonomy of flow. The hypothesis put forward suggested that as arterial pressure was increased, increased filtration of fluid from the plasma at the glomeruli would increase protein and cell concentration of the blood passing through the glomeruli, increasing its

viscosity and thus buffering effects of increased pressure. With reduction in arterial pressure, the reverse effect might be expected to occur. This hypothesis necessarily assumed a linear relationship of glomerular filtration rate to arterial pressure, increases in filtration rate paralleling increase in arterial pressure, and vice versa.

The present investigation has revealed that the above hypothesis is not tenable. Apparently, autonomous renal arteriolar changes are basic to an adjustment of glomerular filtration rate whereby this is kept constant as arterial pressure is decreased, thus precluding changes in blood viscosity. Analysis of the clearance data

TABLE 2. SUMMARY OF RELATIONSHIP OF SODIUM LOAD TO URINARY EXCRETION AND TUBULAR ABSORPTION

	MABP	FILT. RATE	SODIUM			
			Load	Excreted	Reabsorbed	% Reabsorbed
	mm. Hg	cc/min.	mM/min.	mM/min.	mM/min.	
<i>Control</i>						
	148 (124-176)	40 (32-52)	5.77 (4.7-7.7)	0.160 (0.03-0.41)	5.61 (4.6-7.5)	97.3
<i>Experimental</i>						
1	123 (103-142)	37 (32-45)	5.38 (4.47-6.60)	0.074 (0.03-0.126)	5.31 (4.34-6.57)	98.7
2	96 (83-109)	32 (23-37)	4.30 (3.22-5.48)	0.0173 (0.009-0.035)	4.27 (3.2-5.5)	99.5
3	74 (68-82)	21 (11-30)	2.94 (2.8-4.0)	0.009 (0.001-0.026)	2.93 (2.8-4.0)	99.7
4	58 (44-68)	5 (1-8)	0.61 (0.14-0.8)	0.0013 (0.0-0.1)	0.61 (0.3-1.18)	99.7
<i>Recovery</i>						
	136 (119-156)	30 (22-36)	4.10 (3.0-5.0)	0.026 (.008-.057)	4.07 (3.0-5.0)	99.3

Data taken from 5 animals, for one kidney only. Kidney wt., range 41-43 gm. Each level represents the average of 10 urine collection periods. Figures in parentheses are the range of variations.

by the method of Lammport reveals that this maintenance of glomerular filtration rate (and effective plasma flow) is by afferent arteriolar dilatation. It is interesting to note that Forster's data on the rabbit (2) suggest that the constancy of renal blood flow in the face of increased arterial pressure is by afferent arteriolar constriction. These findings together identify the role of the afferent arterioles as a buffering mechanism which counteracts the effects of changes in systemic arterial blood pressure. Beyond the conclusion that this regulation is definitely intrarenal, no further information can be supplied at present as to the exact nature of this buffering mechanism.

The findings in connection with the alterations in the renal clearance mechanism of sodium during graded arterial pressure decrement throw some light on the problem of sodium retention in congestive heart failure. In this condition, due to reduction in cardiac output, glomerular filtration rate and effective plasma flow are decreased, the latter more so than the former (3, 4). Decreased filtration rate is associated with decrease in urinary excretion of sodium.

Our findings lead to the conclusion that as glomerular filtration rate is decreased, the load of sodium to the tubular cells is decreased, with the result that tubular reabsorption becomes more complete at lower filtration rates. In fact, the present data suggest that an actual 'threshold' for sodium exists, below which sodium reabsorption is complete.⁴ The normal kidney offers a load which is above this 'threshold' with the result that small amounts of sodium are normally excreted in the urine. This threshold has been computed for the present series and tentatively set at a value of 3.9 mm. of sodium per minute per kidney (average weight 42 gm.). The load at which sodium excretion begins is delivered to the tubules at a filtration rate of about 25 cc/min., 63 per cent of the control average. This is in no wise to be interpreted as meaning that a 'Tm' (tubular maximum) for sodium exists here, for in the range of the present data tubular reabsorption continues to increase as load is increased, even though accompanied by increased urinary excretion. Higher sodium loads to the tubular cells would be required than attained in the present series to establish the presence of a Tm such as exists, for example, for glucose.

The implication of these findings to the renal mechanism in congestive heart failure is apparent, notwithstanding. Because of reduction of glomerular filtration rate in congestive failure, a smaller load than normal is offered to the tubular cells whose reabsorptive capacity does not appear to be altered. This load is less than the 'threshold' for sodium, and as a result all filtered sodium is reabsorbed and hence retained in the blood by the kidneys. Sodium retention supplies the osmotically active substance needed for the fluid retention which leads to edema formation.

It is interesting to note that when glomerular filtration rate is improved in patients by the use of xanthine diuretics (4) that sodium excretion increases noticeably. This may be assumed to mean that the increase in sodium load resulting from increased filtration exceeds a threshold such as exists in dogs. Likewise, when glomerular filtration rate in patients normally edema-free is reduced by exercise below a critical level of 70 cc/min., sodium retention and edema formation begin (8), also supporting the concept that a threshold exists below which sodium reabsorption is complete.

SUMMARY AND CONCLUSIONS

When arterial infusion pressure to the kidney is gradually reduced by gradual aortic occlusion, clearances of p-aminohippurate and creatinine are well maintained

⁴ 'Threshold' has been defined elsewhere as a critical plasma concentration below which tubular reabsorption of certain filtered constituents is complete, and above which urinary excretion begins. Since plasma sodium concentration remains essentially constant in these experiments, but filtration rate is varied, we employ the term 'threshold' to designate a critical *load* (plasma concentration X filtration rate) below which sodium reabsorption is complete and above which urinary excretion begins.

near control values through a range of about 150 mm. Hg to 100 mm. Hg. Subsequently, clearances decrease as glomerular filtration rate and urine flow cease at about 60 mm. Hg. During this latter phase, creatinine clearances fall more rapidly than the PAH clearances, so that the filtration fraction decreases.

Calculation of renal resistance changes by the method of Lampion indicates that maintenance of renal clearances is due to afferent arteriolar dilatation; efferent arteriolar resistance and post-arteriolar resistance remain essentially constant. This emphasizes the role of the afferent arterioles as a buffering mechanism to maintain blood flow and glomerular filtration in opposition to systemic arterial blood pressure changes.

Renal excretion of sodium decreases as glomerular filtration rate is decreased by aortic occlusion. This is because as filtration is reduced, the sodium load to the tubular reabsorptive mechanism is decreased with the result that tubular reabsorption becomes more complete. The data suggest that a 'threshold' for sodium reabsorption exists at a load of about 3.9 mm/min/kidney below which reabsorption is complete. The normal kidney offers a load somewhat above this threshold so that small amounts of sodium are normally excreted. The significance of these findings as they bear on the problem of renal sodium retention in congestive heart failure is discussed.

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VALIDITY OF PULSE CONTOUR METHOD FOR CALCULATING CARDIAC OUTPUT OF THE DOG, WITH NOTES ON EFFECT OF VARIOUS ANESTHETICS^{1, 2}

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THREE years ago (1) we presented a procedure by which the stroke index of the dog could be estimated from the contour of the aortic pressure pulse. Since, in the dead dog, the contracted and the relaxed aorta showed very similar increments in volume with a given increment in pressure, it was assumed that these volume increments would not be changed in significant degree in the living dog under various physiological conditions. The application of this assumption to the calculation of the stroke index from aortic pressure change proved successful.

To perform this calculation, the arterial bed was divided into four parts, the transmission time to each part being estimated from average pulse wave velocity figures. These respective times were then applied to an aortic pressure pulse to indicate the corresponding pressure increase above diastolic in each part at the end of ejection. The volume uptake of each part was obtained by referring the respective pressure increments to the table of volume change per unit pressure rise for each part, which table was derived from measurements made on dead dogs. To the summated uptakes was added an estimated systolic drainage volume. The stroke indexes, which were the sum of the blood taken up by the distended arteries and the blood drained out through the arterioles during systole, showed surprisingly good agreement with those given by the dye injection technique in 45 cases.

This agreement would imply that even though tone changes might occur in the arterial bed, they did not alter to a significant extent the net volume uptake per unit pressure rise. While, in these 45 cases, we had employed various means to change the stroke index considerably, the experiments were not specifically designed to emphasize conditions where tone change might be assumed to be great. The series has therefore been expanded with that goal in view, and also to test the applicability of the method at high and low blood pressure levels, which had not been adequately determined.

METHODS

A total of 187 comparisons, using 66 dogs, have been made between stroke indexes estimated from the pressure pulse contours, and those given by more direct

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measures. In 61 cases, the dye injection technique⁸ (2) was used. In the other 126 cases, the direct Fick procedure, using oxygen, was employed. Oxygen uptake was recorded from a Sanborn spring-balance spirometer, connected to an intratracheal cannula. Venous blood was collected through a catheter whose tip lay either in the outflow tract of the right ventricle or in the pulmonary artery, as determined by fluoroscopy. In all determinations, a continuous recording of aortic pressure was made, by a sound passed down the left carotid artery into the ascending aorta, connected to an optical manometer.

All dogs were anesthetized during the procedure, the usual routine being the use of 10 mg/kg. morphine and 15 mg/kg. sodium pentobarbital (I.V.). In some cases morphine only was used, and in others ether, or urethane, was employed. In the first 45 cases, already published, various drugs and hemorrhage were used to vary the stroke index. Three general experimental procedures were used for the rest of the comparisons. 1) Dogs were subjected to serial hemorrhages, comparisons being made before hemorrhage and at selected times during the course of the bleeding. In this category are 23 control, and 75 post-hemorrhage determinations. In 36 of the 75 cases, the animals had received 5 mg/kg. Dibenamine⁴ 30 minutes before the start of the hemorrhage. 2) Twenty-nine comparisons were made on dogs after both carotid sinuses had been resected, and both vagi sectioned. Sixteen of these were made during the period of acute hypertension, and 13, when the pressure was being reduced from high levels by hemorrhage. 3) Fifteen comparisons were made during the course of a constant infusion of 80 μ g/min. of epinephrine.

Experience with the method of calculating the stroke index from the pressure pulse, as first advanced, has revealed that it is needlessly elaborate. Uptake of the four arterial beds can be obtained easily and rapidly, while the calculation of the time-pressure areas for the estimation of systolic drainage is cumbersome. In this calculation, outflow is assumed to be proportional to arterial pressure less 20 mm. Hg, and the drainage accomplished after the end of the ejection period taken to be equal to the previous arterial uptake.

Since time is required for the pulse wave to reach the terminal arterioles (T_w), the net effective systole (T_s') for the whole of the arterial tree is represented by the first portion of the aortic pulse only, and has a duration of $T_s - T_w$, where T_s is the total length of systole of the aortic pulse. The effective time of diastole (T_d') would then be $T_d + T_w$, where T_d is the length of diastole of the central pulse. Systolic drainage can then be calculated from
$$\frac{T_s' (P_s' - 20)}{T_d' (P_d' - 20)} \times U$$

where P_s' and P_d' are the mean pressure values during effective systole and diastole, and U is the arterial uptake.

As first described, the time-pressure areas of the numerator and denominator were obtained by dividing the pulse into a series of segments in which pressure change could be regarded as following a straight line. The area of each segment was then calculated on the basis of its being a trapezoid, and the several areas summated.

⁸ The Beckman Spectrophotometer used in this study was obtained through a grant from the Division of Grants, National Institute of Health, U. S. Public Health Service, to Dr. V. P. Sydenstricker.

⁴ The Dibenamine was generously supplied by Smith, Klein and French Laboratories.

Systolic drainage averages about 16 per cent of the stroke volume. An error of 10 per cent in the estimation of drainage represents but a negligible error in stroke volume. A painstaking calculation of the areas of numerous segments is, therefore, seldom justified. We have modified our procedure to obtain the values of Ps' and Pd' as simply as possible, the calculation of time-pressure areas being done only as a terminal step.

In the calculation of the pulse of figure 1, for example, the first step is the recording of Pd , Ps , Ts and Tc , i.e. 106 mm. Hg, 128 mm. Hg, 110 msec. and 480 msec. respectively. The Tw value for a diastolic pressure of 106 mm. Hg being 58 msec., $Ts - Tw$ is 52 msec., which is rounded to 50 msec. The first 50 msec. of the pulse curve is then divided into a convenient number of time-intervals, say 5 of 10 msec. each. The pressure at the diastolic level and at the end of each of these intervals is then recorded, and an average taken. Actually this average is made in terms of

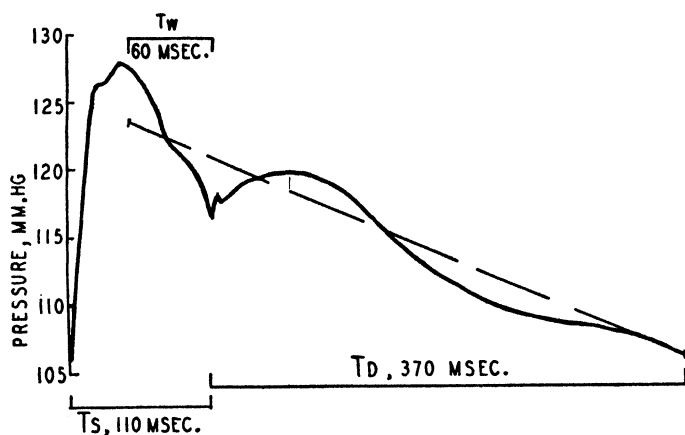


Fig. 1

measuring plate scale units, with a conversion of only the average to pressure units.

For the above pulse, Ps' would be $\frac{106 + 116 + 126 + 127 + 128 + 127}{6} = 122$ mm.

Hg. A straight line is then projected by eye from the end of diastole to the start of $Td + Tw$, such that the pressure values above and below the line are about evenly matched. The average pressure for this interval is then obtained from the values at each end of this line, i.e. $\frac{123 + 106}{2} = 115$ mm. Hg. = Pd' . These values can now be

fitted into the formula: $\frac{50 (122 - 20)}{430 (115 - 20)} \times U$ to give the systolic drainage.

RESULTS

Comparison of Contour with Dye Injection or Fick Stroke Indexes. The deviation of the calculated stroke index from the dye injection or Fick value is expressed in terms of percentage of the letter. In table 1A are shown these percentages as grouped in ascending order of the mean pressure level. In table 1B, the grouping is in ascending order of the stroke index. In neither case is there any consistent trend away from

the reference value. No experimental procedure employed has consistently worsened the fit. We believe, therefore, that the contour calculation will afford a reasonably accurate measure of the stroke index between mean pressure values of 23 and 200 mm. Hg, and that the errors involved are seldom greater than those found when any two measures of cardiac output, e.g. the dye injection and Fick procedures, are compared on the same individual (3). Admittedly, neither the dye injection nor the Fick procedure can test whether stroke indexes calculated from beat to beat are accurate. Both are relatively long-term procedures, the dye injection being more rapid than

TABLE 1. COMPARISON OF STROKE INDEXES OBTAINED BY DYE INJECTION OR FICK PROCEDURES WITH THOSE CALCULATED FROM PRESSURE PULSE CONTOURS

A							B					
NO.	MEAN PRES-SURE	STROKE INDEX, D OR F	STROKE INDEX, CONT.	AV. ERROR	RANGE OF ERROR		NO.	STROKE INDEX, D OR F	STROKE INDEX, CONT.	AV. ERROR	RANGE OF ERROR	
					%						%	
	mm. Hg.	cc.	cc.	± %	-	+		cc.	cc.	± %	-	+
10	23	4.0	3.8	10.8	31	20	10	3.0	3.2	10.0	3	27
10	33	4.6	4.6	10.1	19	27	10	4.0	3.8	13.7	31	18
10	49	8.3	8.1	8.9	11	38	10	5.1	5.5	14.3	30	45
10	57	9.0	8.9	4.8	4	15	10	6.6	6.9	14.9	19	33
10	68	9.0	8.6	9.9	5	22	10	7.7	7.9	13.8	10	41
10	76	13.6	14.0	16.2	13	53	10	8.6	8.8	6.2	1	26
10	83	16.2	16.0	8.6	17	15	10	9.5	9.8	15.9	13	46
10	87	21.5	21.7	9.7	7	21	10	10.6	10.6	10.5	24	28
10	92	24.5	23.6	11.2	20	37	10	12.0	11.8	9.3	24	2
10	96	32.5	32.1	8.0	12	19	10	13.4	13.3	8.1	31	15
10	100	28.8	28.9	9.2	16	20	10	14.8	14.7	10.1	25	14
10	104	26.4	26.5	12.3	23	30	10	16.3	15.9	12.7	35	0
10	108	21.8	21.7	7.7	26	11	10	18.1	18.0	12.9	42	53
10	115	24.3	24.6	10.5	22	24	10	22.6	22.3	11.3	26	14
10	123	23.9	23.9	9.7	50	10	10	26.8	26.4	7.5	50	10
10	131	20.8	22.2	17.5	42	42	10	29.8	30.0	9.8	21	34
10	148	17.7	17.9	13.9	34	33	10	36.3	36.3	8.8	21	13
10	171	16.4	16.8	9.1	21	12	10	43.0	43.3	3.5	7	8
7	211	16.0	15.8	14.3	20	45	7	50.8	51.2	9.1	21	14
Av...		18.1	18.2	10.1				18.1	18.2	10.1		

the Fick. Pulse contours often change during the course of the measurement. While an average of many calculated stroke indexes over the course of the measurement checks with that given by the reference procedure, a single pulse taken at random from the record may be considerably in error.

The calculation has been used by two other laboratories. Huggins, Handley and La Farge (4), in a small series of cases, found agreement with the direct Fick as good as those given here. Duomarco *et al.* (5), using a metered flow into the heart in open-chest dogs, found large discrepancies. We have no evidence to indicate why the technique failed so badly in their hands.

Normal Cardiac Index Values for the Dog. Even when the same procedure of

flow measurement is employed, cardiac index values given for dogs by different investigators have varied quite widely. Wiggers (6) has presented a summary table showing average values ranging from 2.63 to 6.41 l. What seems not to have been appreciated is that the anesthetic employed can explain much of this difference. In the table cited, animals under barbiturate anesthesia show low cardiac index values, those under morphine sedation intermediate values, and those under ether high values. Unanesthetized dogs, as perhaps might be anticipated, show values ranging from the highest to the lowest. In table 2 are shown a series of average values obtained on recently anesthetized dogs, before any extensive experimental procedure had been employed. No claim is made as to comparability of anesthesia levels. Ether was used when entrance into the abdominal or thoracic cavity was desired, and the anesthesia was heavy enough for good muscle relaxation. We do not believe that the

TABLE 2. EFFECT OF ANESTHETICS UPON SOME CARDIOVASCULAR FACTORS IN THE DOG

ANESTHESIA	NO. OF DOGS	MORPHINE DOSE	ANESTHETIC DOSE	PULSE RATE PER MIN.		DIASTOLIC PRESSURE		SYSTOLIC PRESSURE		CARDIAC INDEX		RESISTANCE ¹		LEFT VENTRICLE WORK	
				Mean	S.D. ²	Mean	S.D. ²	Mean	S.D. ²	Mean	S.D. ²	Mean	S.D. ²	Mean	S.D. ²
		mg/kg.	mg/kg.			mm. Hg		mm. Hg		L.				gm. m/sec.	
Morphine	10	10		70	29	74	9	140	24	2.63	0.39	1.81	0.36	4.10	0.41
Urethane	8	10	500	94	38	83	22	129	17	2.77	0.68	1.70	0.47	4.10	0.17
Sodium pentobarbital	47	10	15	107	36	80	15	117	17	2.81	0.75	1.81	0.57	5.90	0.29
	5	10	30	177	35	88	15	116	17	2.63	0.60	2.08	0.80	5.20	0.24
	6	0	30	191	31	129	15	150	17	1.82	0.45	4.00	1.03	4.80	0.18
Sodium barbital	7	0	180	156	35	108	16	133	17	2.24	0.60	3.04	0.80	5.00	0.30
Ether	20	10		126	42	88	15	133	21	3.68	1.02	1.46	0.54	6.84	0.17

¹ Calculated as mean pressure—20 mm. Hg/flow/sec./sq. M. ² Standard deviation.

differences between anesthetic regimes can be attributed to different depths of anesthesia, however. Increasing the dose of barbiturate, or deepening the ether anesthesia makes even larger the differences in flow, resistance and pressure under the two agents.

The values given in table 2 can be grouped into three general categories. The largest cardiac indexes, and lowest resistance values, are seen with ether anesthesia. In general, the deeper the anesthesia, the larger the cardiac index. In the first stages, the increased flow might be attributed to the cardioacceleration which is present in the excitement phase. Later, the heart becomes slow and the pulse pressure becomes very large. The steadily falling resistance denotes a direct dilator action of the anesthetic.

Intermediary flow and resistance values are seen with morphine sedation, or morphine coupled with urethane or a small dose of sodium pentobarbital. While the averages are not significantly different, there is a tendency for the dogs under mor-

phine alone to have lower flows. This is probably directly related to the very slow heart rates. An acceleration is almost invariably accompanied by an increased cardiac index. Atropinization also generally gives an increased flow.

The larger doses of barbiturates give quite a different picture. The immediate response to an intravenous injection is, as is well known, a decline in pressure. This decline is accompanied by an increased cardiac index. This reaction is but temporary and the pressure stabilizes at a level appreciably higher than those found with the other anesthetics. When the barbiturate is given intraperitoneally, the initial hypotensive period may not be seen. The elevated pressure level is the result of an increased resistance, for cardiac index levels are depressed despite the increased heart rate. It is common knowledge, too, that an animal receiving barbiturate in large amounts will often show a gradually developing circulatory failure. In this decline, the resistance remains high, the pulse pressure becoming progressively smaller. The cardioacceleration has been ascribed to a blocking, by the barbiturate, of the efferent vagus action on the heart (7). The only clue we can offer as to the mechanism underlying the increased resistance is that an epinephrine-blocking dose of 15 mg/kg. Dibenamine will lower the resistance, and increase markedly the cardiac index.

SUMMARY

A somewhat modified procedure is given for the calculation of the stroke index of the dog from the contour of the aortic pressure pulse. The quantitative validity of the method has been tested by 187 simultaneous comparisons with the dye injection or the direct Fick techniques. The average error is ± 10.1 per cent, and there is no evidence of a systematic error in the contour calculation. Experiments designed to produce tone changes in the arterial bed did not produce significant change in the accord of the calculated with measured stroke index. It is concluded that the contour method is reasonably accurate within a mean pressure range of 23 to 300 mm. Hg.

The basal cardiac index and resistance values of the dog are dependent in large degree upon the anesthesia employed. Ether seemingly acts as an effective vasodilator, with a resulting large cardiac index, low resistance and moderately low arterial pressure values. Barbiturates, in full anesthetic doses, tend to produce elevated arterial pressure, low cardiac index and high resistance values.

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COMPARISON OF CARDIAC OUTPUT BY THE DIRECT FICK AND PRESSURE PULSE CONTOUR METHODS IN THE OPEN-CHEST DOG

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IN A recent paper (1) the conclusion was drawn from the data presented that the pressure pulse method of Hamilton and Remington for calculating cardiac output (2) is not sufficiently accurate either quantitatively or directionally for use in cardiovascular studies. Their method (1) consisted of measuring the input of blood into the right or left ventricle and calculating the output from aortic pressure pulses. While admitting the procedure was drastic and the dogs deteriorated the viewpoint was expressed that the fundamental requirement of the pressure pulse method was not affected, i.e. "it is only necessary that the blood be ejected into an arterial system with normal and unchangeable volume elasticity constants." The conclusion following from their work is that perhaps as a result of the procedures to which the dogs were subjected the distensibility factors have been altered. If this has occurred the basic assumption of Hamilton and Remington's work has been invalidated. An examination of the pressure pulses shown reveals contours that must be classed as unusual and suggests that possibly some changes in the normal cardiovascular relationships have been induced by the procedures used. The contours may be characterized as showing tremendously large pulse pressures, slow heart rates, very long systolic duration (the majority over 200 m.sec.), steep diastolic slopes despite the very low pressure ranges studied and the slopes show little if any inflection as though the rapid flow rates would continue to very low pressure levels. There is also a relatively high calculated peripheral resistance.

There are at least three possible explanations for such pulses: 1) That contrary to the opinion of Hamilton and Remington distensibility can change markedly in the arterial tree and that in these dogs the aortae were relatively inelastic. This factor could in part explain the straight diastolic pressure descents. 2) In these dogs the peripheral bed was wide open. If this is true, flow should slow quite markedly at pressure values of 20 to 40 mm. Hg (3) and cease at about 20. These curves show no evidence of this. With a high peripheral resistance the cessation of flow should occur around 50 mm. Hg. 3) The aorta was losing blood through some other channel (aortic regurgitation). The meter would not measure such regurgitated blood, whereas the contour method would. Regurgitation would increase diastolic and thus systolic drainage and the resulting stroke volumes would be high.

Regarding the last suggestion, in attempts to utilize the pulse contour method in

humans (4), it was found that patients in chronic congestive failure give falsely high stroke volumes. This phenomenon could conceivably be due to an abnormally inelastic aorta or to regurgitation into a dilated ventricle.

A further reason for uncertainty about the method used by Duomarco *et al.* and the condition of the dogs affecting the volume elasticity constants of the arterial tree lies in the excellent agreement found between simultaneous output comparisons using the dye method (2) and the direct Fick (5) in the intact animal. In the original paper (2) a fairly extensive series of stroke volume determinations were done with an agreement between the two methods of ± 8.2 per cent (average difference). The series of comparisons with the dye has since been extended and in addition a series of comparisons using the Fick method has been done with an agreement of ± 10.1 per cent (6). Huggins, Handley and La Forge (5) using the Fick principle found agreement of the order of ± 12.2 per cent (average difference). In a more recent paper (7) dealing with the effects of various drugs on cardiac output in dogs bled to a mean pressure of 50 mm. Hg and held there for 90 minutes, the cardiac output determined from pressure pulses obtained during the hypotensive period, in every case, compared favorably with published data (8, 9) in which the cardiac output was determined by accepted methods.

For the above reasons the problem was reinvestigated using a different method. In this paper, data are presented comparing cardiac output calculated from pressure pulse contours and the direct Fick method in the open-chest dog.

METHODS

The dogs were anesthetized with sodium barbital, 300 mg/kg. A Hamilton manometer (10) of adequate frequency was used to obtain the pulse contours. The cannula leading to the manometer was inserted into the left carotid before the chest was opened and records made prior to and immediately after opening the chest. The azygous vein was tied and large cannulae, one in the superior and another in the inferior venae cavae, were inserted. The cannulae were connected to a rotameter through a 'y' tube and the blood was returned to the heart through a cannula inserted into the right auricle. The rotameter data will be presented in a separate paper. Chlorazol Fast Pink, 80 mg/kg. was used at the anticoagulant.

For the direct Fick, oxygen consumption was measured by connecting the dog to a McKesson Recording Metabolism machine. The O_2 circuit consisted of a hose leading from the output valve of the metabolism machine and connecting to a 'y' tube on the other end of which was a balloon sealed in a bottle. The remaining end of the 'y' tube was connected to the tracheal cannula. Oxygen moved from the machine through the balloon, through a water valve and into the trachea. A tube connecting the other end of the tracheal cannula to the return valve of the metabolism machine completed the circuit. Positive and negative pressure was applied to the balloon through a 'y' tube in the sealed bottle by suitable connections with a respirator. Oxygen consumption was determined in 5-minute periods. The O_2 circuit was tested for leaks after the dog was killed by running the machine for a minimum period of 5 minutes with the dead dog in the circuit. The blood samples were taken in oiled syringes simultaneously from the femoral artery and the rubber tube just above the

cannula that returned blood to the right auricle. This sample did not of course contain blood from the coronary sinus. The syringes were placed on ice as soon as the blood samples were taken. Blood oxygens were determined by the method of Roughton and Scholander (11). Simultaneous with the drawing of the blood samples, pulse contours were recorded.

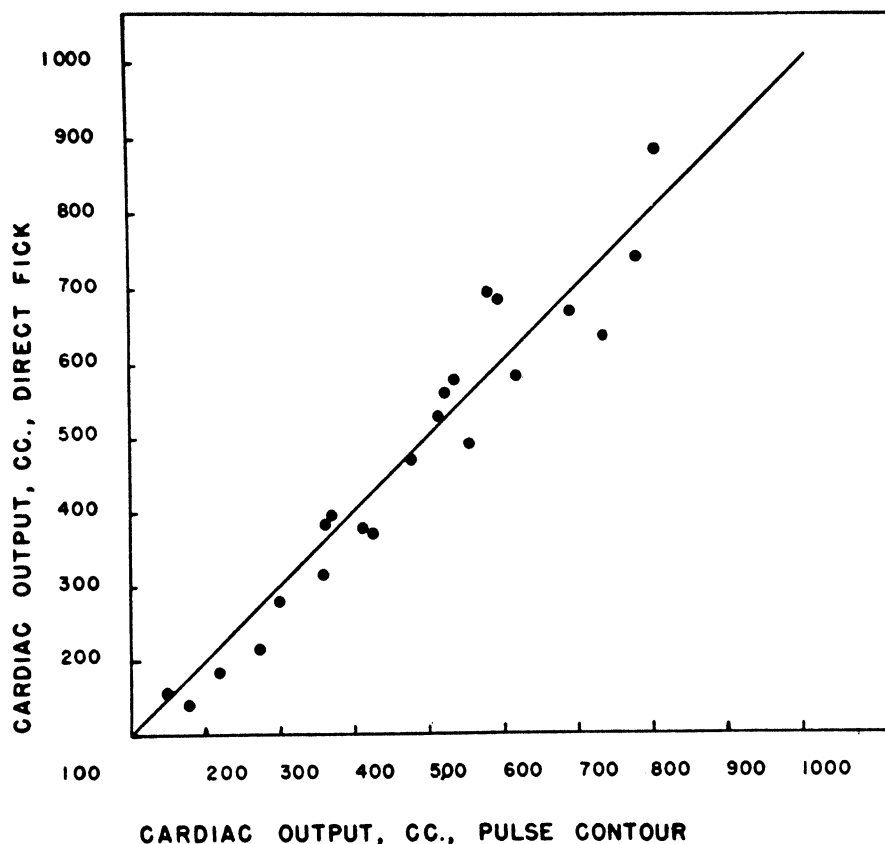


Fig. 1. The solid line represents a perfect correlation.

The cardiac output determined by the pressure pulse method is expressed in m^2 of body surface. This was corrected to give a figure for the individual dog to agree with the Fick data.

RESULTS

A total of 23 simultaneous cardiac output determinations were made on 5 dogs and the data are incorporated in figure 1. Comparing the direct Fick and the pressure pulse data the average difference is ± 12.5 per cent with a range from -13.7 to $+59$ per cent and a coefficient of correlation of $r = +0.985$. While the average difference is ± 12.5 per cent, the pressure pulse method on the average gives values 6.7 per cent higher than the Fick method. This difference can probably be accounted

for by the fact that the pressure pulse method includes the coronary flow while the direct Fick as adapted to this experiment probably does not.

DISCUSSION

The data presented here are obviously not in agreement with that of Duomarco *et al.* (1). They found no correlation between the calculated output and measured input while our data indicate an excellent correlation between the calculated output by the pressure pulse method and the direct Fick. The reasons for the differing results is not clear, however, in no instance were pressure pulse contours observed equivalent to the ones published by Duomarco *et al.* Further, the agreement reported here between the measured and calculated cardiac output indicates that the distensibility factors were unaffected by procedures about as drastic as those they used.

In one instance the pressure pulse value is higher than the direct Fick by +59 per cent. If this determination is eliminated from the data the largest deviation is a +27.1 per cent.

SUMMARY

The method of Hamilton and Remington for calculating cardiac output from pressure pulse contours agrees closely with the simultaneously determined cardiac output by the direct Fick method in open-chest dogs.

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COMPARISON OF THE CONSTANT AND INSTANTANEOUS INJECTION TECHNIQUES FOR DETERMINING CARDIAC OUTPUT

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ONE method of measuring cardiac output involves the use of dyes which are known to remain within the cardiovascular tree for prolonged intervals. Measurements are carried out in two ways, either by the injection of dye at a steady rate over a regulated interval (1), or by a single, rapid injection at the beginning of the determination (2). The constant injection method depends upon the establishment of a concentration plateau when the injected substance is quantitatively diluted by once-circulated blood. The existence of such a plateau has been questioned by Hamilton and Remington (3), and these authors doubt the validity of the constant injection technique. The work reported here has been designed to compare the two methods by doing consecutive determinations on the same animal, using the blue dye T-1824.

MATERIALS AND METHODS

Thirty experiments were carried out on 10 adult mongrel dogs of both sexes, whose weights varied from 11 to 27 kg. Paired experiments were done, an instantaneous injection being followed by a constant injection in each case. Animals were anesthetized initially with intraperitoneal sodium nembutal in doses of approximately 150 mg/kg. and small additional intravenous doses were given as necessary throughout the experimental period. The right external jugular vein was exposed and through a large tributary a no. 8 French ureteral catheter was introduced into the right auricle. A bleeding cannula was inserted into either the right omohyoid or the right common carotid artery.

T-1824 was made into solutions containing either 10 or 15 mg/cc. In the instantaneous injection experiments the dye was injected into the auricular catheter from a syringe calibrated to deliver either 4.60 or 2.65 cc. at the distal end of the catheter. Injection was carried out as rapidly as possible, always being completed in less than two seconds. For constant injections an air pressure device similar to that described by Wiggers (4) was used to give a steady rate of flow. The burette was attached to the auricular catheter through a capillary tube. In these experiments amounts varying from 5.7 to 7.5 cc. of dye were injected over the entire period

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of the determination. The rate was constant for each animal, ranging from 0.3 to 0.4 cc/sec. in separate experiments.

Using a rotating kymograph sampling technique (3), blood samples were collected at measured intervals which were constant in each run and which varied from 0.56 to 0.59 seconds in separate runs. For a cardiac output measurement, 30 such samples of approximately 1 cc. each were collected in tubes, each containing 0.1 mg. of sodium heparin in 0.01 cc. of 0.9 per cent sodium chloride solution. These tubes were centrifuged at 2500 rpm for approximately 15 minutes and a 0.1 cc. aliquot of plasma withdrawn from each. Each aliquot was diluted with 5.0 cc. of 0.9 per cent sodium chloride solution, and its dye concentration determined by measuring its optical density. Measurements were carried out in a Cole-

TABLE 1. COMPARISON OF CONSECUTIVE DETERMINATIONS OF CARDIAC OUTPUT BY CONSTANT AND INSTANTANEOUS INJECTION TECHNIQUES

DOG NO.	WT. IN KG.	CARDIAC OUTPUT		PERCENTAGE VARIATION ¹
		INSTANTANEOUS INJECTION	CONSTANT INJECTION	
		<i>l./min</i>		
1	17.5	3.55	3.45	-3
2	18	3.45	3.15	-9
3	15	3.65	3.35	-8
4	11	2.80	2.50	-11
5	18	2.80	2.80	0
6	16	3.40	3.25	-4
		2.20	3.10	+41
7	27	2.20	2.55	+16
		2.15	2.10	-2
8	18.5	2.35	2.50	+6
		2.25	2.40	+7
9	17.5	3.10	2.65	-15
		2.05	2.25	+10
10	18	2.25	1.90	-16
		2.05	1.95	-5

¹ Measured as percentage variation of constant from instantaneous injection.

man Junior spectrophotometer set at 620 λ , and the samples were compared with standard solutions of dye. These standard solutions were prepared by adding known amounts of dye to tubes containing 5.0 cc. of 0.9 per cent sodium chloride solution and 0.1 cc. of the animal's own plasma (5).

All animals were autopsied, and in every case the catheter proved to be in the right auricle.

The cardiac outputs were determined according to the formulae employed by Hamilton and Remington (3).

RESULTS AND DISCUSSION

Data on the 30 experiments are shown in table 1.

Figures 1 and 2 show two representative pairs of curves. Constant and instantaneous injection curves are superimposed to show time relationships. Graphs of all

15 constant injection experiments show well defined plateaus similar to those shown in these figures.

It is claimed by Hamilton and Remington (3) that since recirculation occurs so rapidly, a true concentration plateau is impossible. It is clear from the work reported here that such plateaus occur regularly, regardless of the time when recirculation

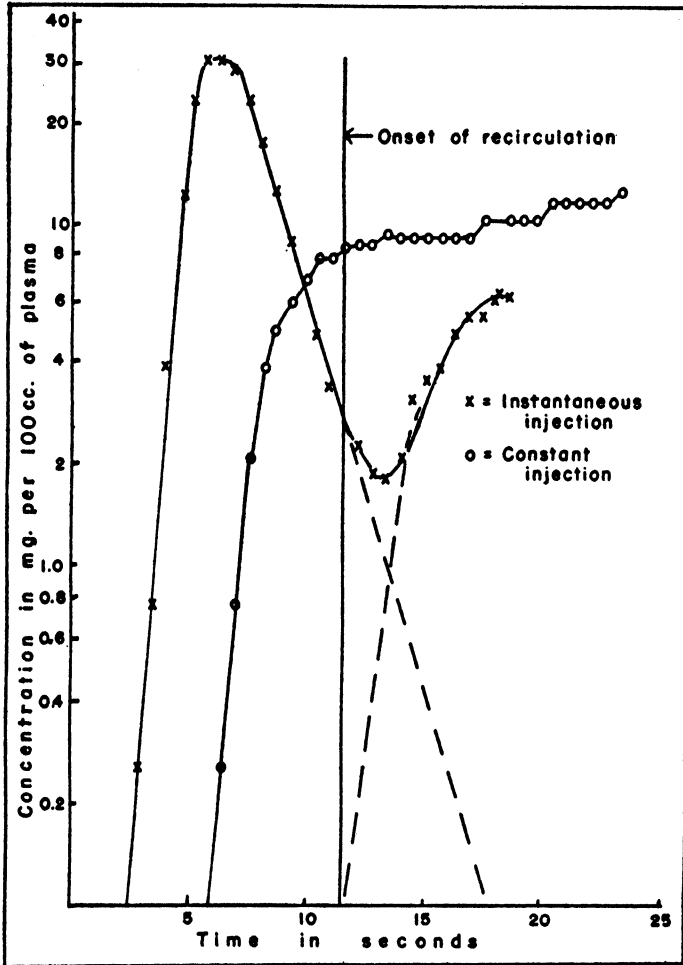


Fig. 1. Doc 2

begins. If the amount of recirculated dye raised significantly the level of the plateau, early recirculation should result consistently in low output determinations by the constant injection method. In this work no such consistently low results have been obtained.

In figure 3 a scatter of the values obtained by the constant injection method as compared with instantaneous injection values is plotted. Examination of the figure and the data in table 1 shows no consistent directional variation between the

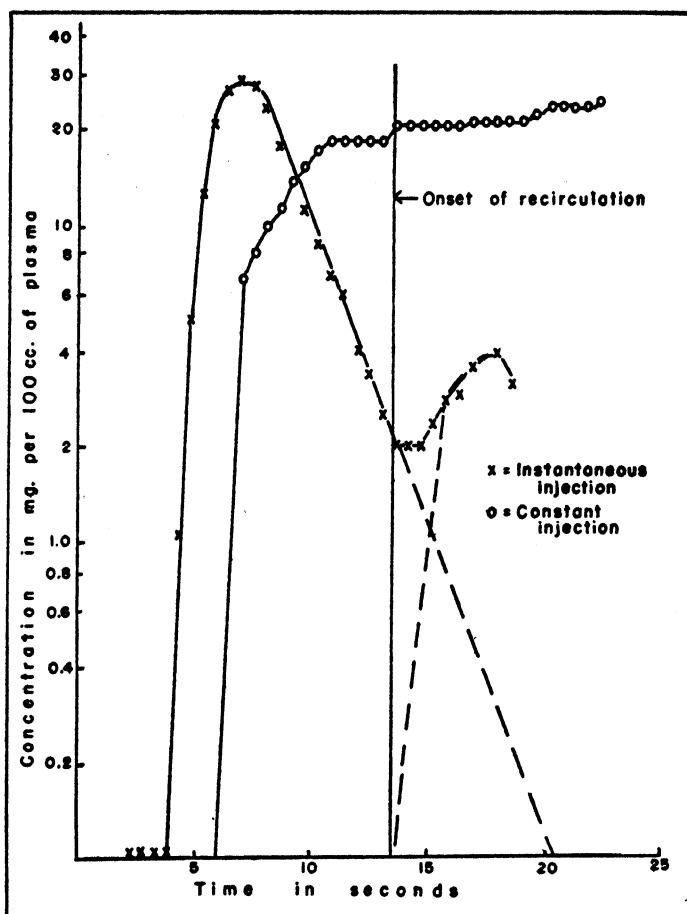


Fig. 2. Dog 10

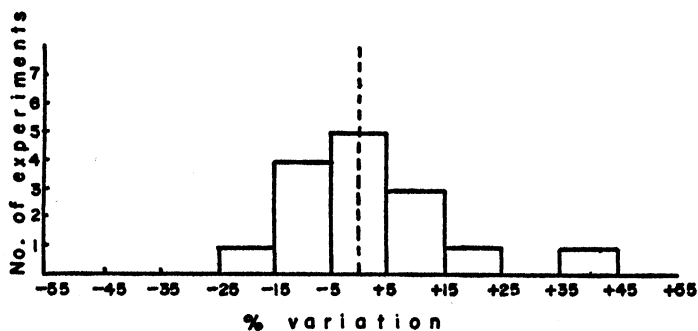


Fig. 3. PERCENTAGE variation of the 15 pairs of experiments

two methods. The variation between the two methods was 10 per cent in this experiment, a figure well within the experimental error of the methods.

SUMMARY

Consecutive measurements of the cardiac output were carried out by the constant and instantaneous dye injection methods. The results of the two methods agreed within 16 per cent in all but one determination. The distribution of the percentage variation between the two methods showed a normal, symmetrical scatter. The constant injection technique for the determination of cardiac output is as valid as the instantaneous injection method.

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CHANGES PRODUCED IN HEMATOCRIT VALUE, HEMOGLOBIN AND PLASMA VOLUME BY REPEATED ARTIFICIAL PNEUMOTHORAX

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ARTIFICIAL pneumothorax has long been employed as a therapeutic agent. Detailed information concerning physiological alterations induced by pneumothorax is largely limited to the respiratory exchange and pulmonary blood flow. There are a few scattered reports of changes in the formed elements of the blood following pneumothorax. Burkner, Ederle and Kircher in 1913 (1) found an increase in the red blood cell count and hemoglobin concentration in dog, rabbit and man as a result of repeated insufflation. Similar results were obtained in the dog by Moog and Pelling (2). Both groups attributed the polycythemic response to anoxia. In man, 3 workers (3-5) described an increased red blood cell count and hemoglobin percentage during repeated pneumothorax, while another (6) found no increase.

The present study represents an attempt to investigate the nature of the reported polycythemia and possible erythropoietic stimulation. Data collected by early workers were confined to measurements of the concentration of formed blood elements and often involved general anesthetics. It was hoped that with the use of the plasma volume determination and other procedures, more information might be obtained.

METHOD

Closed pneumothorax was induced in 13 dogs, weighing 7 to 12 kg., by injecting room air measured in a 100 cc. syringe and delivered through a 16-gauge needle inserted in the fourth or fifth intercostal space. During this procedure the unanesthetized animals were loosely restrained in the supine position. The needle and syringe were connected with a water manometer. The presence of the unobstructed tip of the needle in the intrathoracic cavity was determined by the smooth respiratory fluctuation of the negative pressure developed in the manometer. In order to maintain a reproducible degree of pneumothorax, the quantity of air delivered at each refill was determined by the elevation of intrathoracic pressure. Injections of 100 cc. to 900 cc. every 2 to 5 days on alternate sides were made to produce an immediate average intrathoracic pressure of +20 mm. H₂O (expiration to -40 mm. H₂O (inspiration)). In 2 to 5 days, the corresponding pressures were reduced to approximately -20 mm. and -80 mm. These pressures in the normal dog were -50 mm. and -100 mm. The duration of a course of repeat insufflations varied from 11 to 75 days.

Blood samples were obtained from the jugular vein and femoral artery. Hematocrit percentages were determined in duplicate by the Van Allen tube, oxygen capacity by Grant's method (7), and oxygen content by the Roughton-Scholander technique (8). Plasma volume was measured as described by Gregersen (9) using the blue dye T-1824. The percentage of plasma protein was calculated from the refractive index of plasma determined with Abbe refractometer (10). Reticulocyte percentages were estimated by the method of Osgood (11). An occasional red blood cell count was also made.

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Blood samples were removed at least 48 hours following an insufflation of air in the great majority of cases. This procedure was adopted since the chronic effects were those generally desired.

Pneumothorax was preceded by a control period during which the animals were trained to lie quietly on the board. Blood samples were analyzed every few days for at least three weeks or until the values had stabilized.

RESULTS

Conditions of Animals. An increase in respiratory activity was apparent immediately after insufflation. In 3 animals ventilation was measured quantitatively. It was found that minute volume had increased above that of the control period. The increase was the result of an accelerated respiratory rate and occurred despite a diminished tidal air volume. The percentage oxygen saturation of arterial blood in 18 to 20 measurements made on 5 dogs was within the range of control values. It would appear that the compensatory reactions following the pneumothorax were adequate to prevent anoxic anoxia. Arterial blood pressure measured by direct needle puncture in 4 dogs showed no significant variation from that of the control period.

All animals were maintained in good condition throughout the entire period. Body weight remained rather constant and no untoward effects of the procedure were evident. Since the dogs led a somewhat confined life, no instances of dyspnea on exertion were encountered.

Immediate Effect on Blood. Although the primary purpose of this study was to determine the influence of long continued artificial pneumothorax on the formed elements of the blood, some attention was directed toward any immediate effect. A few measurements were made in 5 of the animals at hourly intervals after the primary insufflation and all results were in substantial agreement. Figure 1 gives the results of a primary insufflation of 650 cc. in a 12-kg. dog. Hematocrit percentage, oxygen capacity, red blood cell count and plasma protein percentage were measured several times during a 2-hour control period and for 4 hours after pneumothorax. Another set of measurements were obtained the next day. As shown in figure 1, no significant change occurred in any values as a result of insufflation. In addition, an intravenous injection of T-1824 was made during the control period. The optical density of the dye in plasma yielded the characteristic slow disappearance curve. It will be noted that no shift occurred following insufflation, indicating a lack of marked fluid movement into or out of the vascular bed.

Chronic Effects on Blood. Eight series of pneumothoraces, lasting 20 to 75 days, were performed using 7 normal dogs. Hematocrit percentage, oxygen capacity, as well as red blood cell count when occasionally done began to rise 3 to 8 days following the institution of pneumothorax. This rise then persisted from 15 to 30 days after which, if insufflations were continued, all values remained at the elevated plateau. If, on the other hand, insufflations were terminated the blood values returned to those of the control period within 6 to 12 days. The average increase in hematocrit values ranged from +4 to +30 per cent above the control figures with an average increase of 19 per cent. An example of the change in hematocrit percentage, oxygen capacity and red blood cell count following pneumothorax is shown in figure 2.

Of the 8 animals in the series in which pneumothorax was produced, 7 demon-

strated definite increases in the *values* of the various physiologic responses being studied. One animal, *number 6*, showed only +4 per cent increase in hematocrit volume, the next lowest being +12 per cent. It is interesting to note that this animal was the only one in which subcutaneous emphysema was noted following insufflations. Air injected into the intrathoracic cavity of this animal soon leaked into the subcutaneous tissue spaces in large quantities. Intrathoracic pressure rapidly returned to normal and the state of pneumothorax was evanescent (table 1).

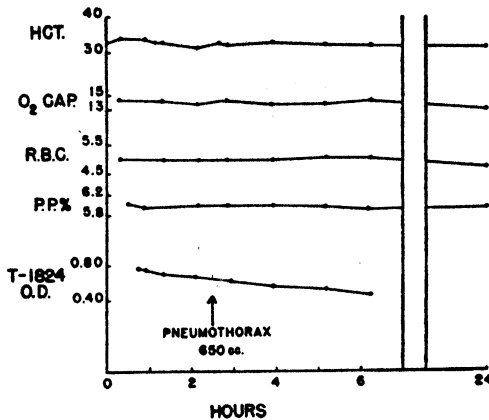
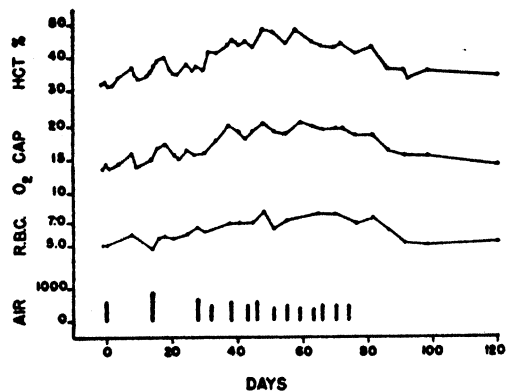


Fig. 1. ACUTE INFLUENCE of a single insufflation on hematocrit percentage, O₂ capacity (vol. per cent), red blood cell count (millions/cu. mm.), plasma protein percentage and optical density of T-1824 in plasma. No significant changes are evident in the concentration of the formed elements and the disappearance rate of T-1824 shows no marked fluctuations.

Fig. 2. EFFECT OF REPEATED AIR INSUFFLATIONS on the formed blood elements (fig. 1). Intervals of 14 days separated the 1st and 2nd and 2nd and 3rd refills. In these relatively long intervals the blood values rose slightly on each occasion. Following the 3rd insufflation, the interval was decreased and the values rose steadily to a maximum.



Erythropoiesis. The rather slow increase in erythrocyte concentrations following a period of insufflation suggested at once an increased erythropoietic activity. To this end, reticulocyte counts were made repeatedly on jugular venous blood of all dogs. Entirely negative results were found. At no time did the range of reticulocyte percentages of the pneumothorax period vary from that of the control.

In order to check this unexpected result, the hemoglobin production during the control period was compared with that during insufflations. The method employed was a modification (12) of the classical 'constant level anemia' of Whipple and Robschey-Robbins (13). Three dogs, one of which was splenectomized, were followed

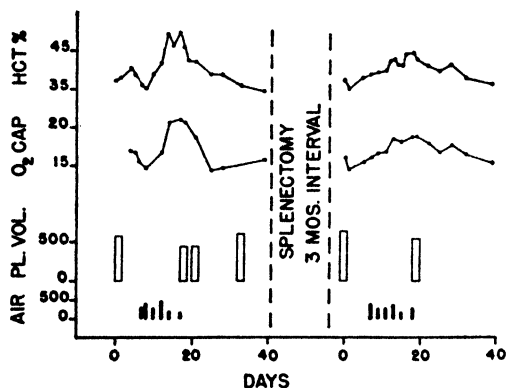
for 4, 4 and 7 months respectively. All animals were bled regularly for 9 weeks in order to exhaust reserve red cells and to establish control hemoglobin production rates. Pneumothorax was then maintained for 21-day periods separated by a control period of similar duration. This process was repeated several times. When comparisons were made of control and pneumothorax periods, two of the dogs showed no

TABLE 1. SUMMARY OF RESULTS FOLLOWING PNEUMOTHORAX

DOG	CONDITION	DURATION OF PNEUMOTHORAX, DAYS	AVERAGE PERCENTAGE CHANGE FROM CONTROL			
			HCT	O ₂ CAP	PV	PP
1	Normal	13	+14	+7	-6	+1
2	Normal	11	+24	+27	-20	+6
3	Normal	14	+25	+24	-20	+11
4	Normal	75	+30	+43	-15	0
4	Normal	36	+26	+25	-28	+23
5	Normal	21	+12	+8	-16	0
6	Normal	12	+4		0	0
7	Normal	50	+19	+22		
8	Splenectomized	55	+10	+12	-20	0
2	Splenectomized	12	+15	+16	-13	0
9	Splenectomized	17	+11		-9	0
10	Splenectomized	17	+10		-5	0

HCT = Hematocrit percentage. O₂CAP = Oxygen capacity (vol. per cent). PV = Plasma volume. PP = Plasma protein percentage.

Fig. 3. EFFECTS OF PNEUMOTHORAX on the same dog before and after splenectomy are shown above. A very definite post-pneumothorax increase on hematocrit percentage and O₂ capacity (vol. per cent) occurred after splenectomy. Plasma volume (cc.) showed an inverse relationship to the other values measured.



significant changes. The third dog, a normal animal, gave evidence of marked hemoconcentration during insufflations but no increase in hemoglobin formation.

Spleen. In a further attempt to explain the increased red cell concentration, the possible rôle of splenic contraction was examined. The customary pneumothorax procedure was carried out on 4 dogs 2 to 3 months after splenectomy. Figure 3 presents the results obtained from an animal given insufflations both before and after splenectomy. Following splenectomy, pneumothorax produced an increase in hematocrit percentage and oxygen capacity. The increase in the 4 splenectomized animals

ranged from +10 to +15 per cent of control values. This is a slightly smaller increase than that shown by the larger control series (table 1). The difference between the two groups is of questionable significance, because of the relatively smaller number of splenectomized dogs and the range of individual variation.

Plasma Volume and Refractive Index. In order to test the possibility of hemoconcentration, plasma volume measurements were made in 9 dogs, both normal and splenectomized. The results indicated that with one exception plasma volume decreased in all animals following pneumothorax. The average change from control ranged from 0 to -28 per cent. The single animal showing no change was the same one whose hematocrit value increased only +4 per cent over the control figure. The period of maximal elevation of the concentration of blood elements after insufflation coincided with that of minimal plasma volume. Figures 3 and 4 provide examples of

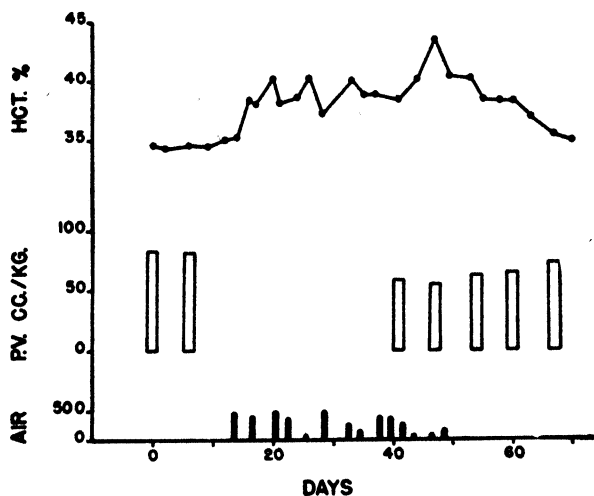


Fig. 4. INFLUENCE OF REPEATED INSUFFLATIONS on hematocrit percentage and plasma volume (cc/kg. body weight) is shown. The inverse relationship between these two values is evident.

the relationship of plasma volume and hematocrit value and table 1 gives a summary of the data.

The number of grams of protein per 100 cc. of plasma was calculated from the refractive index of plasma during plasma volume determinations. Of the 11 series of measurements on 9 dogs, 8 showed no change following pneumothorax. Three, however, increased +6, +11, and +23 per cent, above control values (table 1).

DISCUSSION

Any increase in value of the formed elements of the blood following pneumothorax required from 3 to 8 days to become apparent and 2 to 4 weeks to reach a maximum. It is of interest to note an immediate hematocrit elevation has been reported by Henry and his group (14, 15) in both cat and man within 30 minutes during breathing against a resistance of 20 to 120 mm. Hg. Increase in intrathoracic pressures in the present study never exceeded 5 mm. Hg and usually were much less.

The absence of demonstrable reticulocytosis during the phase of increasing hematocrit, oxygen capacity and red blood cell values negates erythropoietic stimulation.

In addition, the failure of periods of pneumothorax to cause an increased hemoglobin production during 'constant anemia' substantiates this conclusion. Earlier workers (1, 2) believed the polycythemic response to be the result of an increased blood cell production caused by anoxic anoxia. Under the conditions of the present study, neither anoxic anoxia nor erythropoietic stimulation was found.

The post-pneumothorax concentration of formed blood elements persisted after removal of the spleen. If splenic contraction plays a part during pneumothorax its influence is negligible.

The decrease in plasma volume appears to be the most likely explanation of the increased hematocrit value in 9 of 10 animals during the insufflation periods as shown in table 1 and an example is presented in figure 4. Direct determination of the circulating red blood cell mass would be desirable but was not performed. Calculation of total blood volume and red blood cell mass from plasma volume and hematocrit percentage was made during the control and pneumothorax periods. The change in total blood volume ranged from +1 to -16 per cent of control values with an average of -10 per cent. Red cell mass values showed a large degree of variation and the change from control ranged from -10 to +25 per cent with an average of +4 per cent.

From the data presented, it would appear that the increased value of the formed blood elements following pneumothorax is largely a manifestation of hemoconcentration. Fluid of unknown composition is lost from the vascular bed under the stress of increased intrathoracic pressure and the changes associated with it. Although some variation existed in the percentage of plasma proteins, the fluid lost from the blood vessels carried protein with it. The site of departure is not known, but the pulmonary capillaries are those subjected to the greatest trauma and therefore likely to permit leakage. On the other hand, the increased intrathoracic pressure might tend to counteract this process.

A difference in the compartmentation of the thoracic cavity of man and dog exists, yet similar increases in hematocrit values have been reported in both species (1-5). Whether all the changes occurring in dogs as a result of pneumothorax take place in man has not been determined. It is of some interest, however, to speculate on the possible rôle of hemoconcentration during therapeutic pneumothorax.

SUMMARY

Artificial, closed pneumothorax was maintained in a total of 13 unanesthetized dogs for periods of 11 to 75 days by repeated insufflations of room air. Respiratory compensation was adequate and anoxic anoxia was not present. In a few days the hematocrit, oxygen capacity and red blood cell count values rose above those of the control period and reached a maximum in 2 to 4 weeks. At the end of the pneumothorax period, these values returned to the control level in about a week.

No evidence of erythropoietic stimulation was obtained since reticulocyte percentage and hemoglobin production remained within the control ranges. In addition, the presence of the spleen is not essential to the hematocrit increase. Plasma volumes decreased as the hematocrit values increased and this inverse relationship was main-

tained throughout the course of the procedure. Plasma protein percentage showed no consistent change.

It is concluded that the increased values of the formed elements of the blood during pneumothorax is a result of hemoconcentration caused by loss of fluid from the vascular bed.

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DEVELOPMENT OF TURBULENCE IN FLOWING BLOOD¹

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THE conditions which lead to turbulence in flowing blood are of hemodynamic interest for several reasons: *a*) turbulence is generally believed to be an important factor in the production of murmurs; *b*) calculation of the work of the heart depends upon the nature of flow in the central arteries (1); *c*) a study of the development of turbulence yields information important to the understanding of laminar flow in small blood vessels which offer the principal resistance to flow of blood in the peripheral circulation.

Reynolds (2) showed that the transition from laminar to turbulent flow in tubes depends on the dimensionless expression $Re = \frac{\rho \bar{u} r}{\eta}$ where

$$\begin{array}{ll} \rho = \text{density} & \eta = \text{viscosity} \\ \bar{u} = \text{average velocity} & r = \text{radius of tube} \\ & Re = \text{Reynolds' number} \end{array}$$

The Reynolds' number represents the ratio of inertial force to viscous force; it is the condition for mechanical similarity of flow around geometrically similar objects. The inertial force per unit volume is proportional to $\frac{\rho \bar{u}^2}{r}$, while the viscous force per unit volume is proportional to $\frac{\eta \bar{u}}{r^2}$. It is evident that as the flow increases, the inertial force increases faster than the viscous force. The transition from laminar to turbulent flow occurs when the inertial force becomes so great that the viscous force is no longer able to damp stray disturbances in the fluid. When no effort is made to minimize these disturbances in homogenous fluids, this transition occurs at Reynolds' numbers of 1000 to 1100 (critical Reynolds' number).

It has generally been assumed that the flow of blood, like that of homogeneous fluids, would become turbulent at a Reynolds' number of about 1000. Since blood is a heterogeneous system of variable viscosity, this assumption is not justified *a priori*.

The only experimental measurements of the turbulence point for blood appear to be those of Müller (3) who has employed conventional hydrodynamic methods to determine the critical Reynolds' number for ox blood flowing in glass tubes. Müller has shown that the relations between pressure and flow are such as to indicate a transition from laminar to turbulent flow at Reynolds' numbers in the range 700–1000. Our own experiments were begun without knowledge of Müller's results and were designed to correlate pressure-flow data with a study of cellular orientation during the development of turbulence. We planned to demonstrate the turbulence point in two ways: *a*) by conventional hydrodynamic procedures and *b*) by an electri-

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cal method based on observations of Velick and Gorin (4). These authors observed that the electrical resistance of flowing blood, measured in the direction of flow, was less than that of blood at rest. They showed this phenomenon to be a result of orientation of the cells with their long axes parallel to the flow stream. The non-conducting cells, when oriented, occupy less of the cross-sectional area. They thereby permit more current to flow, much as a Venetian blind, when opened, will admit more light. We decided to measure electrical resistance in the direction of flow simultaneously with pressure and flow, anticipating that electrical resistance would rise when the cellular orientation was disturbed by turbulence.

METHODS

A diagram of the experimental apparatus is shown in figure 1. Two flow tubes of dimensions shown in the figure were used; each tube was provided with 2 pressure taps, the first tap being placed at a distance of 150 radii from the entrance of the tube in order to obtain 95 per cent of the parabolic velocity distribution for laminar

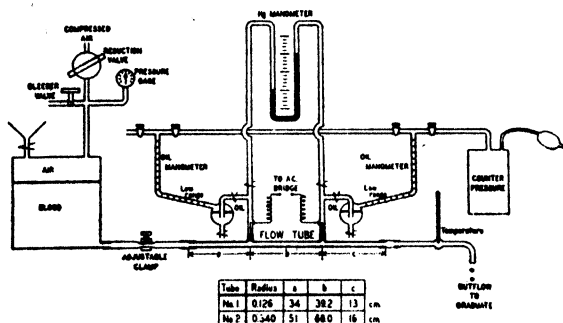


Fig. 1

flow (8). This arrangement eliminated the need for applying kinetic energy corrections. The average radius of each tube was determined by the mercury-weight method and by measuring the electrical resistance, between the pressure taps, of saline of known specific conductivity (5, 6).

The pressure drop between taps was measured by 2 oil manometers at low pressures and a U-tube mercury manometer at high pressures. The oil manometers were provided with inclined scales which could be read to the nearest 0.02 cm. H_2O over a range of 0 to 1.8 cm. H_2O . Vertical scales were provided for intermediate pressures. The mercury manometer was used for higher pressures up to 600 mm. Hg; corrections were applied to allow for the weight of saline within the conduits leading to each arm of the U-tube (mercury) manometer. Flows were measured with graduated cylinders and a stop watch. Temperature of the blood was determined from a thermometer inserted near the outflow. Electrical resistance in the axis of flow was measured between platinum electrodes (fig. 1) inserted into the pressure taps. The measurements were made with an A.C. bridge operating at 1 to 3 kc. Reactive components were balanced out capacitatively using an oscilloscope as a null indicator.

The overall hydrodynamic characteristics of the apparatus were checked by measuring the pressure-flow relations of water and computing the viscosity from Poiseuille's Law. The flow of water became turbulent at a Reynolds' number of 1080 ± 40 .

Bovine blood was obtained from the local abattoir, isotonic sodium citrate and heparin being used as anti-coagulants. NaCN (about 1 mm/l.) was added to inhibit oxidative metabolism and thus prevent reduction of the hemoglobin. It is our impression that the cyanide acted as a preservative and delayed hemolysis. Before each measurement the pressure reservoir was shaken to mix the blood; settling of the red cells at zero flow was detectable as a slow progressive increase in electrical conductivity. Ordinarily this settling was negligible even at low flows; samples taken from the outflow at intervals during each experiment showed no significant changes in hematocrit.

RESULTS

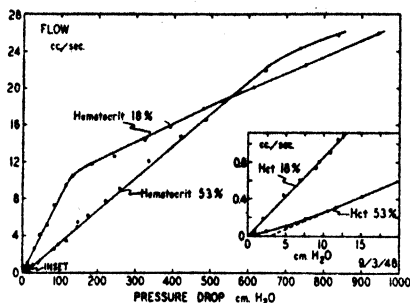
At low flows the apparent viscosity, calculated from Poiseuille's Law, was found to decrease with increasing flow. This anomalous effect is discernible in the inset of figure 2 as a convexity of the pressure-flow curve toward the pressure axis; it is shown in detail in figure 3 for bloods of different hematocrits. It is clear from these results that anomalous flow occurs in large tubes as well as in capillary tubes in which the phenomenon was first demonstrated by Hess (7). At intermediate and high flows the apparent viscosity approached a constant value (fig. 3).

Turbulence was indicated hydrodynamically by a pronounced bend in the pressure-flow curve as seen in figure 2. A more sensitive index of the transition point may be obtained by plotting the dimensionless friction coefficient, $\lambda = \frac{\Delta P}{\frac{1}{2} \rho \bar{u}^2} \cdot \frac{r}{l}$ against the apparent Reynolds' number on a logarithmic scale as shown in figure 4. With this method of analysis all points fall on the same straight line, regardless of the nature of the fluid or the dimensions of the tube, so long as laminar flow exists. At the turbulence point there is a sharp upward deflection of the curve following which all points fall on a second straight line characteristic of turbulent flow (8). It is seen that the transition point for blood occurred at an apparent Reynolds' number of approximately 1000 for both tubes in the 2 examples illustrated in figure 4. The results of all our experiments with bloods of different temperatures and hematocrits are summarized in table 1. The critical apparent Reynolds' number averaged 970 ± 80 .

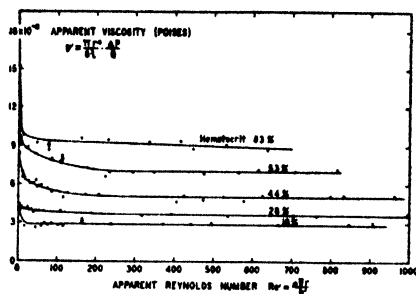
The effects of temperature on the pressure-flow relations of blood of constant hematocrit are illustrated in figure 5. It is seen that the flow at which turbulence begins progressively increases with reduction in temperature as would be predicted from the increased viscosity. This results in a curious paradox: it is seen that in certain pressure ranges above turbulence the flow of cold blood is greater at a given pressure than that of warm blood. A similar condition exists in bloods of different hematocrit; thus, blood of high hematocrit may present less resistance to flow than blood of low hematocrit (e.g. in the upper pressure ranges illustrated in figure 2). This paradoxical behavior may be of physiological significance in the turbulent

flow of blood through stenotic apertures. In this case a high hematocrit might actually be advantageous in minimizing resistance to blood flow through the stenosed region.

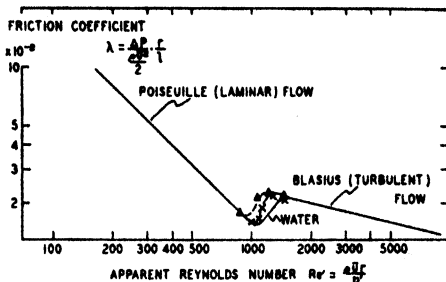
The electrical resistance decreased with increasing flow at low Reynolds' numbers paralleling the changes in apparent viscosity in this range. (Compare figs. 3 and 6).



PRESSURE — FLOW DATA
SHOWING ANOMALOUS VISCOSITY AND DEVELOPMENT OF TURBULENCE
IN BOVINE BLOOD AT 27°C
 $r = 0.126$ cm. $l = 39.2$ cm.



VARIATION OF BLOOD VISCOSITY WITH REYNOLDS NUMBER
Bovine blood at $30 \pm 4^\circ\text{C}$ $r = 0.126$ cm. $l = 39.2$ cm.



DEVELOPMENT OF TURBULENCE IN FLOWING BLOOD
Bovine blood, hematocrit 29% at $29 \pm 1^\circ\text{C}$
x — Tube No. 1. $r = 0.126$ cm. $l = 39.2$ cm.
Δ — Tube No. 2. $r = 0.340$ cm. $l = 68.0$ cm.

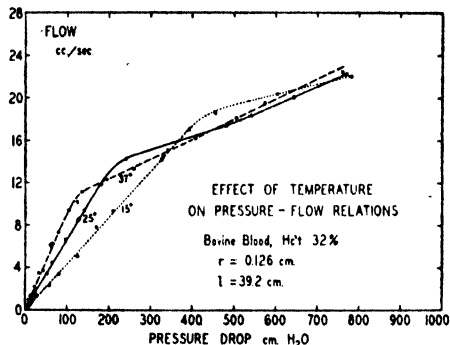


Fig. 2 (upper left). PRESSURE-FLOW DATA showing anomalous viscosity and development of turbulence in bovine blood at 27°C ; $r = 0.126$ cm., $l = 39.2$ cm.

Fig. 3 (lower left). VARIATION OF BLOOD VISCOSITY with Reynolds' number. Bovine blood at $30 \pm 4^\circ\text{C}$; $r = 0.126$ cm., $l = 39.2$ cm.

Fig. 4 (upper right). DEVELOPMENT OF TURBULENCE in flowing blood. Bovine blood, hematocrit 29% at $29 \pm 1^\circ\text{C}$.

x — Tube No. 1. $r = 0.126$ cm., $l = 39.2$ cm.

Δ — Tube No. 2. $r = 0.340$ cm., $l = 68.0$ cm.

Fig. 5 (lower right). EFFECT OF TEMPERATURE on pressure-flow relations. Bovine blood, hematocrit 32%; $r = 0.126$ cm., $l = 39.2$ cm.

Presumably these changes in electrical resistance result primarily from orientation of the cells in the flow stream. At zero flow the (non-conducting) cells are randomly oriented and the electrical resistance is high; as flow progresses, the cells orient to present least hydrodynamic resistance and both the apparent viscosity and the electrical resistance diminish in regular fashion toward constant values.

At the turbulence point the electrical resistance did not rise but, contrary to expectation, remained constant up to a Reynolds' number of 2500 which was the

highest we could conveniently obtain with our apparatus. This is shown in figure 6 for bloods of various hematocrits. The contrast in behavior at turbulence between

TABLE I. CRITICAL APPARENT REYNOLDS' NUMBERS OF BOVINE BLOOD IN GLASS TUBES OF DIFFERENT BORE AND AT DIFFERENT TEMPERATURES AND HEMATOCRITS

TUBE RADIUS r	HEMATOCRIT	DENSITY ρ	TEMPERATURE	APPARENT VISCOSITY JUST BELOW TURBULENCE η (poises)	CRITICAL APPARENT REYNOLDS' NUMBERS $\frac{\rho \bar{u} r}{\eta}$
cm.	%	gm/cc.	°C.		
.126	18	1.033	27	.029	900-1090
.126	21	1.035	24	.033	890-1010
.126	28	1.043	29	.037	1000-1090
.126	40	1.048	34	.047	1015-1035
.126	44	1.054	27	.051	960-1070
.126	53	1.054	28	.070	820-990
.126	32	1.040	14	.057	815-945
.126	32	1.045	26	.036	850-1050
.126	32	1.048	37	.026	960-1140
.340	18	1.032	27	.030	845-1000
.340	29	1.042	29	.040	785-1110
.340	45	1.051	28	.047	880-1080
Mean					970 \pm 80

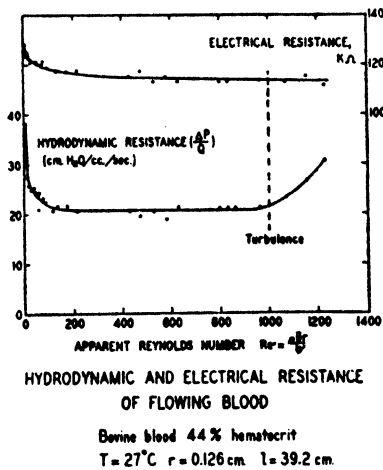
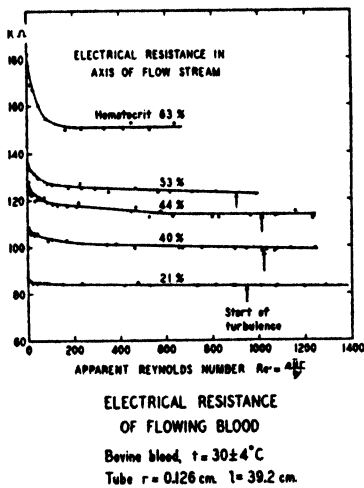


Fig. 6 (left). ELECTRICAL RESISTANCE of flowing blood. Bovine blood, at $30 \pm 4^\circ\text{C}$.; $r = 0.126$ cm., $l = 39.2$ cm.

Fig. 7 (right). HYDRODYNAMIC AND ELECTRICAL RESISTANCE of flowing blood. Bovine blood, 44% hematocrit; at 27°C .; $r = 0.126$ cm., $l = 39.2$ cm.

hydrodynamic resistance, defined as $\frac{\Delta P}{Q}$, and electrical resistance is clearly shown in figure 7. We can only conclude that the red cells remain oriented despite the turbulence demonstrated simultaneously by hydrodynamic methods.

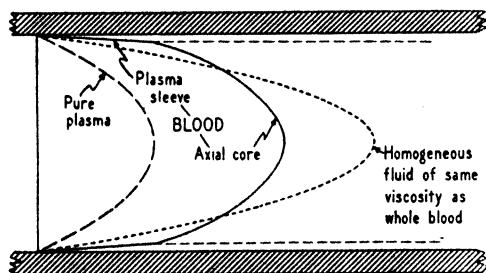
DISCUSSION

The constancy of electrical resistance of blood in which an orientation effect is electrically demonstrable at low flows indicates that the cells remain oriented in turbulent flow. It is difficult to see how they could remain oriented if they did not occupy a region of the flow stream which was free of turbulence. It follows, then, that turbulence must occur in a region which is free of cells.

It is well known that, in vessels of small caliber, the cells tend to accumulate in the axial stream (9, 10). Presumably this same phenomenon occurs in larger vessels. Surrounding the axial core of cells and plasma is a 'peripheral sleeve' of essentially cell-free plasma. The constancy of electrical resistance at turbulence indicates that laminar flow is maintained in the axial core, and that turbulence is confined to the peripheral sleeve.

If this hypothesis is correct, we may think of the apparent viscosity of blood in laminar flow as the resultant of two viscous resistances in parallel: *a*) the viscosity of plasma alone in the peripheral sleeve and *b*) the viscosity of cells plus plasma in the

Fig. 8. VELOCITY DISTRIBUTION CURVES just before transition to turbulent flow. The velocity distribution curve for this blood was calculated on the assumption that turbulence occurred when the average velocity of the peripheral sleeve plasma equalled the average velocity of plasma alone at turbulence in the same tube. If this assumption be accepted then the values of radius and viscosity of the axial core can be calculated from the observed viscosities of whole blood and plasma; substitution of these values in Newton's laws of viscous flow yield the composite velocity distribution curve shown.



axial core. This is represented in figure 8 for laminar flow just below the transition to turbulence. As flow is further increased, a critical average velocity of the peripheral plasma is eventually reached beyond which the inertial force in the peripheral sleeve alone is sufficient to maintain turbulence. In the axial core, however, the viscous stabilizing influence of the red cells is still great enough to prevent this turbulence from spreading beyond the peripheral sleeve. We thus have a two-phase system, with turbulence in the (thin) peripheral sleeve proceeding simultaneously with stabilized viscous flow in the central core.

The hypothesis that blood flows as a two-phase system has several implications of theoretical and practical importance:

1. Application of Newton's Law of Viscosity to this two-phase system yields the relation $\eta' = \frac{\mu}{1 - \alpha^2 \left(1 - \frac{\mu}{\eta_0}\right)}$ where η' = apparent viscosity of whole blood;

μ = viscosity of plasma; η_0 = viscosity of central core, and α = fraction of cross-sectional area of tube occupied by the central core. The value of α at turbulence has not been established with certainty but it is probably not less than 0.85 under the

conditions of our experiments (see legend to fig. 8). This estimate yields a minimum value for η_0 which is about 10 times the viscosity of plasma.

2. Doubt is cast on the technique used by Ralston and Taylor (1, 11) in determining the character of flow in glass or lucite tubes inserted into the aortas of dogs and cats. These authors injected India ink into the left ventricle or proximal aorta, and observed the appearance of streamlined filaments of ink in the tubes. This conclusion, valid for water, is not justified for blood in which core flow may be laminar while peripheral sleeve flow is turbulent. From the point of view of calculating the kinetic work of the heart it is clear from figure 8 that the velocity distribution of blood flow cannot be considered a continuous parabolic distribution based upon its apparent viscosity as would be justifiable in a homogeneous fluid.

3. The concept of flowing blood as a two-phase system may have an important bearing on the problem of peripheral resistance. The energy required to maintain laminar flow of homogeneous fluids is greatest in the periphery where the rate of shear is highest. In a two-phase system where the axial core viscosity is considerably greater than that of the peripheral sleeve, this difference between peripheral and central rates of shear is greatly accentuated (fig. 8). It would appear, therefore, that in a two-phase system most of the energy required to maintain flow is dissipated in the peripheral sleeve, the axial core being carried along somewhat like a log in the center of a stream. It is suggested that this pattern of energy dissipation may apply to blood flowing in the arterioles which present the major resistance to flow of blood in the peripheral circulation.

SUMMARY

The development of turbulence in bovine blood flowing through medium bore glass tubes is described.

Turbulence was found by conventional hydrodynamic methods to occur at an apparent Reynolds' number of 970 ± 80 . This value was independent of tube size, concentration of red cells and of temperature (table 1). At very small Reynolds' numbers the apparent viscosity decreases with increasing flow (fig. 3). Anomalous flow of blood is therefore not restricted to capillary tubes but is detectable, with refined methods, in large tubes at low rates of shear.

Electrical resistance, measured in the axis of flow, was found to diminish with flow in a regular manner paralleling the variation in apparent viscosity (figs. 6 and 7). Evidently the non-conducting cells, in orienting to diminish hydrodynamic resistance to flow, also present less cross-sectional area opposing the flow of electric current. Contrary to expectation, the electrical resistance remains unaltered by the establishment of turbulent flow (figs. 6 and 7) even at Reynolds' numbers up to 2500. This suggests that the cells remain oriented and that turbulence occurs in a region free from cells.

The facts are consistent with the hypothesis that blood flows as a two-phase system comprising a peripheral plasma sleeve and a central core of plasma plus cells (fig. 8). Turbulence may develop in the peripheral plasma phase while laminar flow and cellular orientation continue undisturbed in the axial core. The results are discussed with reference to a) calculation of the kinetic work of the heart, b) the

flow of blood through stenotic apertures and c) the nature of flow in the peripheral arterioles.

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